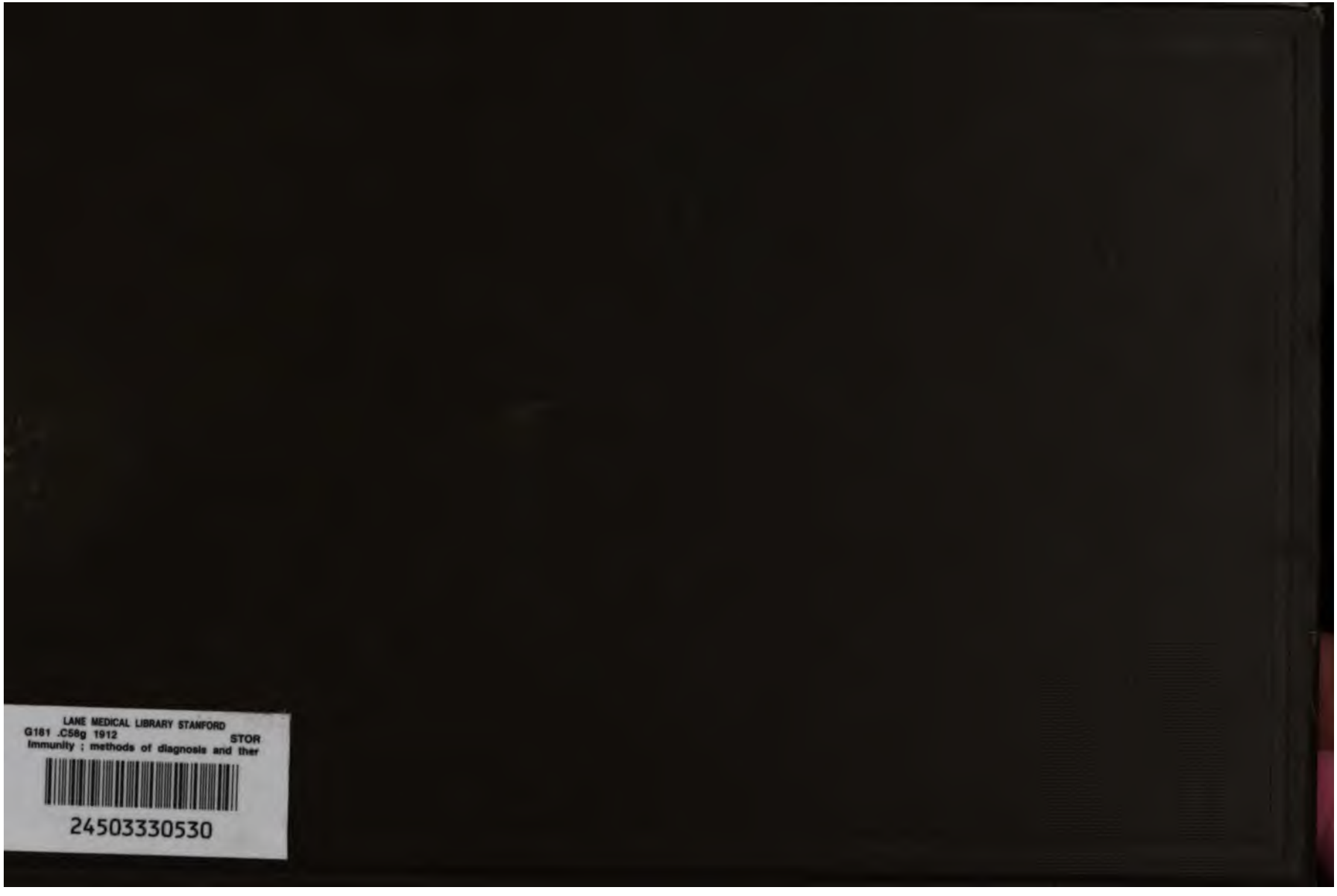

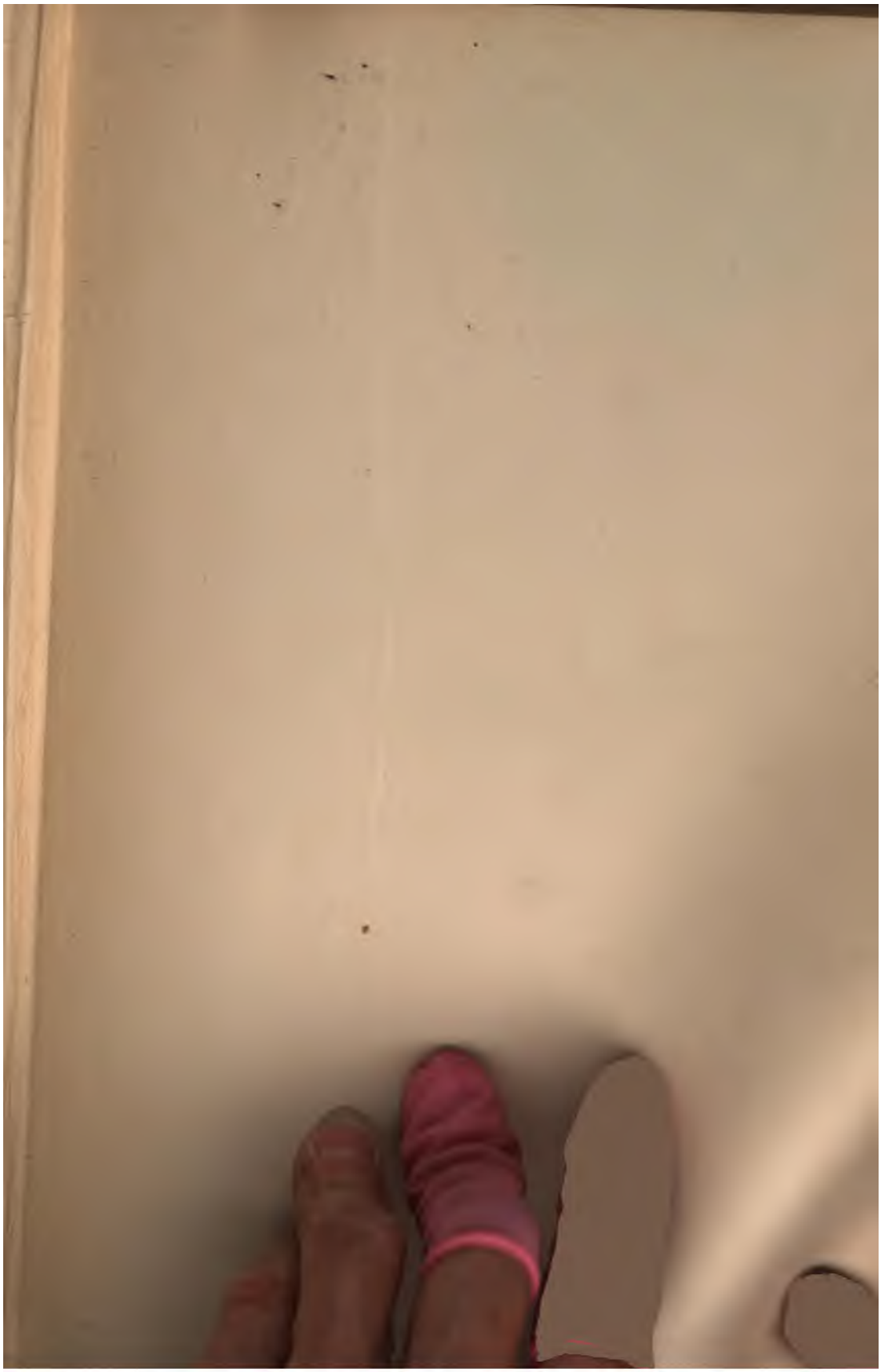

IMMUNITY

METHODS OF DIAGNOSIS AND THERAPY
AND
THEIR PRACTICAL APPLICATION

CITRON

GARBAT

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METHODS OF DIAGNOSIS AND THERAPY

AND

THEIR PRACTICAL APPLICATION

BY

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TRANSLATED FROM THE GERMAN AND EDITED

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TO

PROFESSOR FRIEDRICH KRAUS

AS EVIDENCE OF DUE HONOR AND THANKFULNESS

THIS BOOK IS DEDICATED

BY THE AUTHOR

ON THE OCCASION OF THE OPENING OF THE NEW

II MEDICAL DIVISION

PREFACE TO THE GERMAN EDITION.

This book is to serve a purely practical purpose. The methods of serum diagnosis, on account of their growing clinical significance, are constantly stimulating greater interest in all branches of medical science. While giving instruction in this subject, I realized that it would be of great help to both the medical student and physician if they possessed a short text-book which would review in a purely critical form the various methods of immunity diagnosis, especially those relating to tuberculosis and syphilis.

The two systems of *Kolle* and *Wassermann*, and *R. Kraus* and *Levaditi* are doubtless the standards on the subject in German medical literature. On account of their size and price, however, these volumes come to be sought only by the specialist.

It was therefore my aim in this book to so present the subject of immunity that the general medical man, who is even slightly acquainted with laboratory work, can learn the details of the various reactions and their significance. In selecting the different methods, I have taken up those which are used in the clinic for diagnostic, therapeutic, or prophylactic purposes. In addition there are herein included certain fundamental considerations of questions on immunity which for the present are only of theoretical interest, but which owing to the rapid development of the subject, may soon become of practical importance.

I have endeavored especially to place before the reader a critical review of the results of the various methods. In the description of technical details, the original articles of the author have been selected; modifications having been considered, only provided they exhibit distinct advantages over the original method.

I here wish to express my thanks to my teacher Prof. Wassermann, under whose guidance and stimulus I gained my laboratory experience; also to my chief Prof. Kraus whose clinical genius proved to me the practical importance that this subject of immunity commands.

To the publishers as well, whose kind coöperation in all my plans as regards publication and illustration, greatly simplified my work, I extend my heartiest appreciation.

JULIUS CITRON.

NOTE BY THE AMERICAN EDITOR.

The study of "Immunity," once of merely theoretical interest and purely scientific importance, is to-day no longer such. A realm of practical considerations, considerations which are constantly coming up and enlisting the attention of the busy practitioner have little by little supplanted those phenomena at one time vaguely understood and mostly taken for granted. Gradually have the uncertainties so long dominating and obscuring an intelligent comprehension of the subject been cleared away; mistakes explained; and hypotheses re-established as proven facts. The methods employed for the necessary investigations have naturally improved and increased with such extreme rapidity that a severe task presents itself to one who desires to separate the more from the less valuable ones. It was therefore with extreme satisfaction that I greeted the opportunity of bringing out an English edition of this working hand-book on the various, but most essential methods used in the applications of "Immunity." The author of this volume has, by his exhaustive research and extensive practical experience as a teacher, treated his field with such fulness and preciseness of detail that it is of value not only to the laboratory student, but also to the clinician.

Its already favorable reception in Germany will, it is hoped, be extended to it in America, especially by those whose lack of familiarity with the German language has kept this work beyond their reach.

The chapter on vaccines has been slightly revised and elaborated to conform more closely with the most recently advocated methods of Sir A. E. Wright, to whom the editor is indebted for his experience. Otherwise there has been no need to alter the original text, with the exception that here and there some features which may be of special interest to the English reading public, have been inserted.

I wish in the present connection to express my deep thanks to my teacher Dr. Citron for offering me the privilege of this undertaking, and to the publishers, Messrs. Blakiston & Co., without whose hearty coöperation this would have been impossible, my sincere appreciation.

A. L. GARBAT.

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IMMUNITY.

CHAPTER I.

Introduction.—Definitions of Immunity and Antibody.—The Law of Specificity.—The Necessity of Control Tests.

The diagnosis of infectious diseases can be approached in several ways. In addition to the aid obtained from clinical signs such as the course of the temperature, the changes in the various organs, the exanthemata, etc., the finding of the specific etiological agent of the disease, or the specific antibodies developed by the reaction of the organism are of equal or even greater importance. The course of an infection depends not only upon the nature, the number, and the virulence of the infecting agents, but also upon the behavior of the infected body. *One must consider a disease as the result of the interaction of both of these factors without necessarily being able to attribute the various symptoms to either the one or the other.* Although the general reaction of the organism is varied, it can nevertheless be shown that in spite of even individual differences, the characteristic bacteria and their products bring about a distinct symptom-complex which is usually concomitant with a significant defence on the part of the organism. The means which the body employs in this protection are cellular and humoral in nature. In fact, there is a group of infectious diseases in which the cellular reaction predominates, and another in which humoral changes are pre-eminent; and between these extremes are various intermediate forms. Thus the constantly changing picture of tuberculosis always shows the tubercle as its typical product of cellular reaction; similarly leprosy and syphilis have their peculiar cellular changes. More difficult, however, to recognize by the unaided eye or even the microscope are the finer biological alterations which take place in the body fluids during the course of infectious diseases. Here, special methods are necessary to detect and differentiate the various humoral changes which occur for the main part, in the blood serum. As is known at present, the humoral as well as the cellular immunity reactions are not limited to infectious diseases, but also express normal physiological and pathological conditions. With the conception of Ehrlich's side-chain theory the bridge of understanding for the humoral reaction was built, and

it at once became evident how the physiological phenomena of nutrition and production of energy are identical in their nature with processes which under pathological circumstances lead to the formation of anti-infectious bodies. In an analogous and no less ingenious manner, Metschnikoff has shown that the same cell group of mesenchymal origin which the organism stations against bacterial invasion has physiological and physio-pathological functions to fulfill in the whole animal scale. In the lower animals, these cells aid in the metamorphosis of the body structure, thus leading to the disappearance of entire organs. In the female, they aid in the involution of the uterus after labor, while in the aged, they destroy the nerve cells in the senile atrophied nerve centers or finally as chromophages turn the hair gray. *The border-line between the physiological and pathological status is biologically not sharply demarcated.* It is one single chain of manifestations which possess numerous transitional phases. As the methods of serum diagnosis can prove reactions much finer even than those accomplished by chemistry, their application has not been limited to the chapter on infectious diseases.

By their means also, proteids, even though manifest in minutest traces, can be differentiated. Similarly, the secret of blood relationship has begun to be unravelled; and there is a possibility even of solving the problems of metabolism.

Closely associated with serum diagnosis is the serum therapy. Even though the general application of the latter is not as widely developed as that of the former, it must be remembered that through this medium diphtheria has been transformed from a fatal to a combatible disease, and incidentally made the name of Behring immortal. To-day, attempts are constantly being made to treat other bacterial and toxic diseases by specific therapy and it is to be hoped that success will soon be met with.

The study of serum therapy and serum diagnosis is undertaken in various ways. It is comparatively simple to learn only the purely technical details. All large laboratories have trained assistants for the performance of certain reactions or groups of reactions with absolute precision. Although as we have said, they do such work as assigned to them, with accuracy, they are nevertheless far from a thorough understanding of the subject of serum diagnosis. Unfortunately this blind method of procedure has recently been advocated to an alarming extent. In addition, the practical success which the Wassermann reaction has met with, has inculcated the desire in certain schools of physicians, for the carrying out of this test alone, and thus to become independent of the use of large laboratories. To meet this demand, short courses have been established and the serum diagnosis of syphilis taught with lightning rapidity. That such a state of events is absolutely injurious is clearly evident. It is impossible for one to be a specialist in a certain reaction and at the same time be ignorant of the other

phases in the study of immunity. Unreliable and erroneous results are the inevitable outcomes of such unscientific work.

The plan followed in this book consists in taking up all of the important principles and methods of immunity, even though at present some may attract no direct practical attention. The principle of the now widely important Wassermann reaction had been described years previously by Bordet and Gengou, but merely from a purely theoretical standpoint. Only with the development of the Wassermann test, did it attain its practical importance.

Conception of Immunity.

To start systematically, it is necessary, primarily, to understand certain terms frequently employed. First, the word immunity, requires explanation:

After an individual has recovered from an infectious disease, he passes into a state where he is less or even not at all susceptible to the same infection, although no macroscopical, microscopical or chemical change can be shown to have taken place in his system. This condition is one of immunity. And as the body itself by its own struggle with the invading bacteria has brought about this immunity, it is known as "active immunity." Jenner and Pasteur have employed this mode of immunity acquired spontaneously with the overcoming of an infection in their principle of prophylactic vaccination. The exact nature of this active immunity is only partially understood. It can be shown, however, that the individuals thus actively immunized have within their organism reaction bodies of a specific nature directed against the infecting elements and their poisonous products. These reaction bodies which circulate mainly in the blood serum, are known as *Antibodies.*

The antibodies are of different classes depending entirely upon their varied forms of activity. While some, such as the agglutinins and precipitins have the property of grouping their respective invading agents into small clumps or precipitates without, however, at the same time embracing protective powers, there are other antibodies which act, essentially, for the defense of the organism whether by neutralizing the poison of the bacteria (antitoxin) or by destroying the bacteria (bacteriolysin), or so altering the bacteria that the latter can be more easily destroyed by the cells (bacteriotropin, opsonin). The last three types of immunity can be designated respectively, as antitoxic, bactericidal, and cellular immunity. Naturally there are many intermediate forms. It is very probable that besides these well recognized forms of immunity there may be others, still unknown. Cellular immunity must surely have a far greater range of importance than is at present ascribed to it. There is, no doubt, a distinct cell immunity which acts without the aid of any serum substance and is known as "Tissue Immunity" ("histogene" Immunität).

If the serum of an animal which has been immunized and containing

antibodies, is injected into another normal but non-immunized animal, the latter acquires the power of being immune against the specific infective agent. In this case the immunity was not established by direct cell activity on the part of the animal, for the organism remained passive, and had, as it were, immunity thrust upon it. This form of immunity in contradistinction to "active immunity" is designated as "passive immunity."

The forms of immunity thus far mentioned were all "acquired" either by the spontaneous recovery from the infection or the artifical transmission of the curative antibodies. In contrast, however, to this "acquired" immunity there is a "natural" immunity by which is understood that some animal species are not at all susceptible to certain infections. Thus man has a natural immunity against a group of diseases markedly fatal for some of the lower animals, *e.g.*, chicken-cholera and hog-cholera. That this natural immunity is almost always cellular in character is undeniably true; and the most important form of this natural armament against infection is the powerful leucocyte, capable of engulfing and destroying the invading enemy. In other words, phagocytosis.

Finally one should speak of a "local" and "general" immunity, meaning to express thereby the different resistance and susceptibility that various organs of the same individual display; and also of a "relative" and "absolute" immunity in order to differentiate quantitatively a transitory immunity from one that is of long duration.

Conception of Antibody.

Another term very often employed is "antibody." This, as has already been explained, is a name used to designate the specific bodies which the organism produces as a reaction against the infecting agents and their toxic products. Antibodies are also formed when animals are injected with foreign proteids not of bacterial origin, such as the blood of a different species of animal, egg albumin, etc. In order that these antibodies may be obtained, the substances employed must enter the system "parenteral," *i.e.*, some way outside of the gastrointestinal tract.

In older literature the terms antibody and protective body were used synonymously. That is decidedly incorrect, inasmuch as not all antibodies possess the power of protection and not every actively immune organism, demonstrable antibodies. Furthermore, antibodies as the bacteriolysins which are generally considered to have protective powers, and correctly so too, can exist in a system in large numbers without necessarily rendering that organism immune.

As an example of how complicated various chapters in the study of immunity can be, will be clearly evidenced by a few of the author's experiments with the hog-cholera bacillus. Rabbits rendered actively immune by inoculation with extracts of hog-cholera bacilli possess a serum which when injected into an animal of a different species as the guinea-pig, will render the latter passively immune. If, however, the serum is injected

into another animal of the same class (another rabbit), no protective power is transmitted. In other instances it was shown that the rabbit which was being treated with the purpose of active immunity was in reality never immune, as it always succumbed when injected with living bacteria even though its serum contained bodies which were perfectly able to passively protect guinea-pigs against the same deadly infection.

Just as it is incorrect to consider an antibody and protective body as one and the same thing, it is equally erroneous to deny the existence of protective bodies, because their presence cannot be demonstrated by a certain method of laboratory examination. It must be kept in mind that there are still many unsolved problems in the subject of immunity, and that therefore only the positive findings should be the basis for drawing conclusions.

In order to learn the nature of these antibodies attempts have been made to isolate them chemically. Thus far all such trials have been unsuccessful. It is even uncertain whether these so-called antibodies are definite chemical entities. Only the effects of the serum as a whole are known, and the ingredients in it to which these activities are attributed are thought of as antibodies. For didactic purposes antibodies, as antitoxins, agglutinins, etc., will be spoken of in this book when the antitoxic or agglutinating properties exclusively, are meant.

The Law of Specificity.

In spite of the individual differences which are ascribed to the various classes of antibodies, there is one quality possessed by all—their specificity. To explain this by a rather crude example, may be mentioned the fact that typhoid antibodies will give their various reactions of immunity only when these are performed with the typhoid bacillus, and cholera antibodies only when performed with the cholera vibrio. Substances which lack this essential property of specificity cannot be considered antibodies, although they may fulfill all other requirements. There are indeed limitations to this fast rule, but these will be considered subsequently. For the present the following can be taken as a fixed fact; namely, that every true antibody is absolutely specific, and that all substances or bodies which are not specific cannot be real antibodies. *The law of specificity is the fundamental principle of serum diagnosis.* As soon as the specificity of a reaction becomes doubtful, its diagnostic importance suffers greatly. In the following pages, therefore, the question whether or not a reaction is specific will be repeatedly discussed, and it will be the aim in every way possible, especially by the use of control tests and experiments to outline the limits of this specificity. Here, even at so early a stage of the discussion, the absolute necessity of these control tests must be urged, even though it may appear superfluous to the beginner, when for apparently simple experiments controls are performed which consume more time than the actual diagnostic test itself. Probably also the desire will arise, and perhaps be satisfied, to omit these control experiments. This done, notwithstanding of a possibility to obtain for even a long time, per-

fectly good results, it cannot be too often or too emphatically impressed upon all workers in immunity methods, that the only guard against mistakes and failures in diagnosis is necessarily found in control tests. And especially in doing research work, the latter are indispensable. For, experimental work which involves reasonable possibilities, or has any pretension toward plausibility warrants no true scientific conclusion without the employment of such tests.

The author has made it a rule, whenever new findings in serum diagnosis are published, always to look for the given control experiments. If these are insufficient, then no matter what the contents may be, the value of the research is slight, for all its claims only may, but not necessarily must, be correct.

CHAPTER II.

Laboratory Equipment.—General Technique.

Although some of the tests of serum diagnosis are comparatively simple and can be performed in one's office or even at the bedside, in most instances a laboratory equipment is essential. This of course does not at all imply the necessity of such elaborate apparatus as one is accustomed to find in our present up-to-date bacteriological or serological laboratories where a great deal of complicated research work is done. For the practical application of

Fig. 1.—A room in the laboratory of the Royal Institute of Berlin for the study of infectious diseases.

serum diagnosis, as employed at the hospital or in private practice, an outfit much less costly is perfectly sufficient. As regards the question of a room, the selection of one with two windows, allowing the entrance of sufficient light, is indispensable. At the same time, however, some arrangement should be made in connection with the windows in order that the direct rays

of the sun be prevented from striking one's desk. Strong sunlight may weaken or even destroy the virulence of cultures, or bring about many changes in sera. Even diffuse daylight should not be considered as entirely inert. A general rule to be remembered is never to expose any biological reagent, be it a bacterial culture or any form of its derivative, a serum, or any other substance to daylight any longer than is absolutely necessary. If this dictum is followed, one will avoid many a difficulty.

To conform with this idea, it is wise to have upon the table a small closet into which the cultures and sera can be placed for the time that they are being used. Such a convenient receptacle can be made out of a large cigar box, painted black inside and out, with its lid replaced by a small black curtain.

The table or desk at which one works should be near the window, and covered with filter-paper, upon which should come a glass or asbestos plate. Instead of a wooden table it is certainly more elegant, but costlier to have a top plate of glass. Upon the table there should be a Bunsen burner, a microscope, a lamp for microscopic work at night, a dish filled with sublimate or cresol into which the infected substances, old cultures, used pipettes and graduates are placed.

It is very convenient to have running water and a hood in the same room. Still neither of these is absolutely necessary. As for larger apparatus—must be mentioned, a thermostat, a mechanism for shaking, a dry sterilizer, a good autoclave, a water-bath, an instrument sterilizer, a water or electrical centrifuge, an ice chest, a closet for instruments and glassware, and finally animal cages of the kind that are easily cleansed.

FIG. 2.—Instrument (*After Czaplewski*) for the standardization of platinum loops.

As for instruments and glassware the following are required: scalpels, scissors, forceps, glass-cutter, sterilizable syringes of various sizes, graduates of 10, 25, 100, and 1000 c. cm. capacity each, pipettes of 1 c. cm. with 1/100 divisions and pipettes of 10 c. cm. with 1/10 divisions, a sterilizable pipette retainer, Erlenmeyer flasks, Petri and Kolle's dishes, test-tubes, dark glass flasks, ordinary water glasses, funnels, glass tubes of various sizes, and test-tube racks. Furthermore, a platinum needle and a platinum loop are required. For making a loop of a definite size, and one which can always be referred to, the small instrument devised by Czaplewski is of great help. It consists of four round metal bars of 1, 2, 3, and 5 mm. in diameter around which the platinum wire can be twisted in order to make a standard loop (Fig. 2).

All instruments and glassware used for serum work should be perfectly clean. It is best to have all the glassware plugged with non-absorbent cotton, and sterilized by dry heat. It is never advisable to clean the glassware with strong acids, alkalies or other strong chemicals. If this has been done, the chemicals must be thoroughly removed by washing, as the slightest trace may interfere with the accuracy of some tests.

All used glassware should at once be placed into a disinfecting solution.

For this purpose, lysol, lysoform and cresol solutions are highly recommendable. Sublimate is less efficient because it coagulates albumins and thus may lead to plugging of pipettes which may have contained blood rests. If highly infectious material has been examined, it is best to place the entire disinfectant solution containing the used glassware into the autoclave, sterilize it there, then wash the supply thoroughly with soap, dry and resterilize it by dry heat for 1 to 2 hours at 120° C.

The Technique of Inoculation.

Both for serum diagnosis and serum therapy, the serum is required from animals which have been artificially immunized against the bacteria or their products of secretion. Almost without exception, this immunization is produced by injecting the animal with the infectious virus. The method of inoculation is either *intravenous*, *intra*peritoneal, or *subcutaneous*.

Intravenous Injection.

The technique of *intravenous injection* varies somewhat with different animals. In *rabbits*, the veins running along the outer margins of the ears are most suitable. The assistant sits upon a chair, holds the hind legs and body of the rabbit tightly fixed between his knees and thus has his hands free to steady the rabbit's ears. Another method consists in placing the rabbit upon the table

FIG. 3.—Intravenous inoculation. (*After Uhlenhuth.*)

and firmly holding him there while the injection is made (Fig. 3). The ear is first struck gently with the fingers and washed with alcohol and xylol. If the hair is very long, it should be clipped. If the vein running along the outer margin of the ear is exceptionally small, it can be made more prominent by compressing it between the thumb and index finger at the root of the

ear. No force should be used with the injections; the fluids should be allowed to flow into the blood stream very slowly. Glass syringes, or such as can be sterilized easily, are preferable. Air bubbles are to be carefully guarded against in order to exclude the danger of air embolism.

If infectious material is used for injection, it is advisable in such instances, to place a small piece of cotton moistened in alcohol or carbolic around the point of union between the needle and the barrel of the syringe to prevent the possible escape of any fluid which usually occurs at this point.

After inoculation is completed, the needle should be quickly withdrawn, a small piece of non-absorbent cotton placed upon the needle puncture and compression applied. If non-virulent bacteria or albumin is injected, the bleeding may be almost instantly controlled by firmly squeezing the vessel above the puncture wound with the edge of one's finger nail.

In guinea-pigs intravenous inoculation is more difficult, as here there are no large superficial veins. The Jugular or Iliac vein is therefore chosen, and must be dissected free. It is not necessary to tie off the vessel, but the wound should be firmly compressed by means of clean gauze or cotton.

Intracardial Injection.

Morgenroth has substituted the simpler method of *intracardial* inoculation. The point of maximum pulsation of the heart to the left of the sternum is made out by palpation and a thin sharp needle is inserted at the specified area. The spurting of blood indicates that the needle is within the heart. Thereupon the already filled syringe is carefully fitted on to the needle and the contents slowly injected. The syringe is then detached from the needle and blood is again allowed to spurt out in order to be absolutely convinced that the needle is still in the heart. It is next quickly withdrawn. By this method it is possible to inject about 11/2 c.c. directly into the blood stream.

In *dogs, sheep, goats, horses,* etc., the intravenous injection is given into the jugular vein directly through the skin which must be thoroughly shaved, cleaned and disinfected. Compression by the finger makes the vein more prominent.

In dogs the popliteal vein is frequently selected. In man the intravenous injection is given into one of the veins on the anterior surface of the elbow joint.

Several general rules are to be observed when giving intravenous inoculations. First of all, only *small* quantities of fluids should be injected; secondly, the temperature of the fluids for injection should not differ from that of the body; thirdly, substances that are strongly hemolytic may produce marked disturbances or even sudden death of the animal; fourthly, if an animal is to be frequently inoculated it is best to puncture the vein for the first inoculations as far peripherally as possible and give each subsequent injection more centrally, for very often thrombi are formed at the site of inoculation.

Intraperitoneal Injection.

INTRAPERITONEAL injection is employed most frequently among rabbits and guinea-pigs. The main danger associated with this method is possible injury to the intestines; but by heeding the following advice, this can be prevented. The animal should be fixed or held head down. In this position, the loops of intestines tend to sink toward the diaphragm. This is further helped along by gentle downward massage over the abdomen thus leaving an area, above the bladder, which is sometimes free from intestines. Another protective measure, consists in using a blunt canula which can be made by breaking off the sharp point of the needle. As it is at times difficult to pierce the skin with this blunt instrument, it is advisable to previously make a minute incision through the cutis and subcutis with a sharp pair of scissors and pass the needle through this small opening. The needle should not be plunged directly into the peritoneal cavity, because at the withdrawal, the injected fluid easily escapes through the punctured opening. First, it is inserted subcutaneously upward, in the long direction of the animal; then the hand is raised and the needle forced horizontally forward through the peritoneum, thus leaving the opening in the peritoneum at a different level than the one through the muscles and fascia, thereby making the escape of fluid more difficult. One readily realizes that he has gone through the peritoneum by a relaxation of the reflex abdominal rigidity (Fig. 4).

FIG. 4.—Intraperitoneal injection of rabbit. (*After Uhlenhuth.*)

For the intraperitoneal injection in guinea-pigs, *Friedberger* has devised a procedure which is very satisfactory and furthermore does away with the necessity of an assistant. It can also be employed in Pfeiffer's test for the removal of exudates from the peritoneal cavity. The giunea-pig is allowed to creep into the breast pocket of the laboratory gown until its head and thorax are inside of the pocket. Its hind legs are grasped between the middle and ring fingers of the left hand and flexed on the back, thus giving a free exposure of the lower parts of the abdomen (Fig. 5).

Subcutaneous Injection.

SUBCUTANEOUS inoculation is the simplest of all methods. A fold of skin is elevated between the thumb and index finger of the left hand and the needle plunged into the subcutaneous tissue. In rabbits and guinea-pigs the skin of the back or abdomen is chosen, as the subcutaneous tissue here is not tense. In goats, sheep, and horses the skin of the neck and shoulder region is preferred.

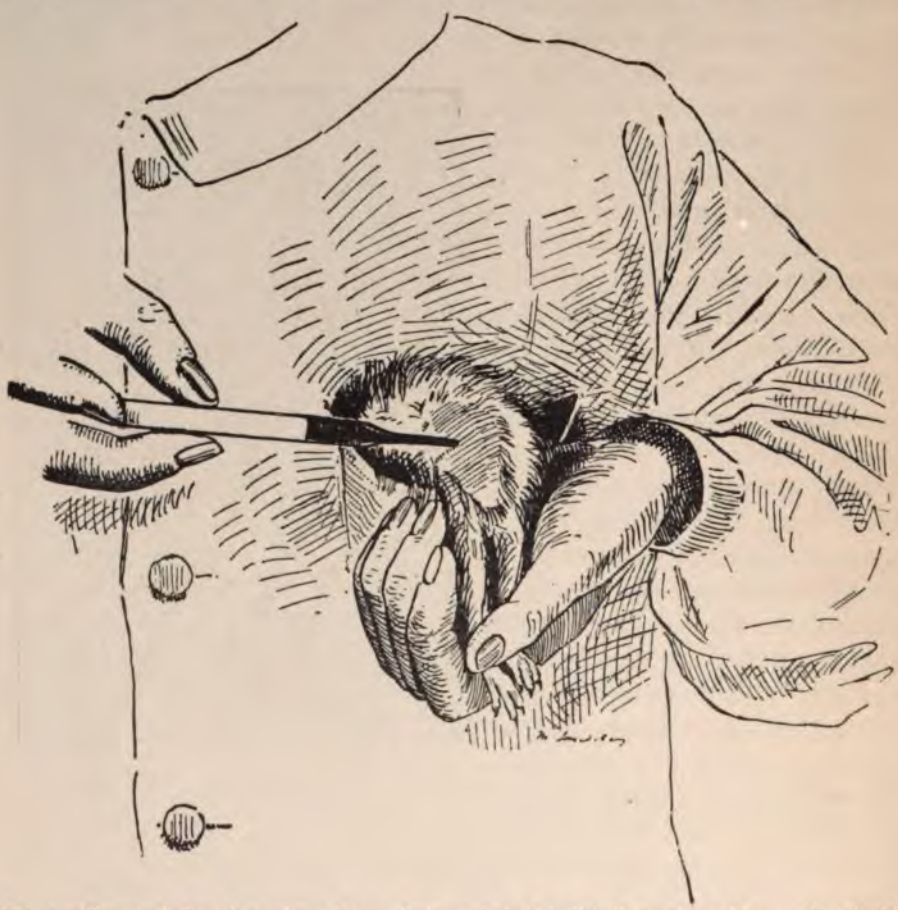

FIG. 5.—Removal of peritoneal exudate. Guinea-pig held in Friedberger's position. (*Original.*)

The skin of the back and abdomen is to be avoided because following the injection edema frequently arises, which may extend to the lower extremities and thus interfere with locomotion.

If abscesses arise after subcutaneous injection, they should be opened, washed out with lysol solution and dressed with iodoform.

The Methods of Obtaining and Preserving Serum.

Venesection or venous puncture is the method best adapted for obtaining blood from animals. The veins employed for that purpose are those which

have already been mentioned in connection with intravenous injections. A simple, large, hollow needle is all that is required. Suction with a syringe is superfluous. Only in Morgenroth's method of removing blood directly from the heart of guinea-pigs is aspiration necessary. In rabbits enough blood can be collected by making an incision into the vein along its long axis, with a sharp knife, or by dividing the vein transversely with the scissors. The blood thus collected is not absolutely sterile.

In man, if only a small quantity of blood is required, it can be obtained from the finger or ear. If, however, a larger amount is necessary, puncture of one of the veins in the bend of the elbow with the Strauss canula is resorted to. It goes without saying that this area must be properly disinfected with soap and water, ether, alcohol or sublimate. Wright's method for collecting moderate quantities of blood will be reviewed in the chapter on opsonic studies.

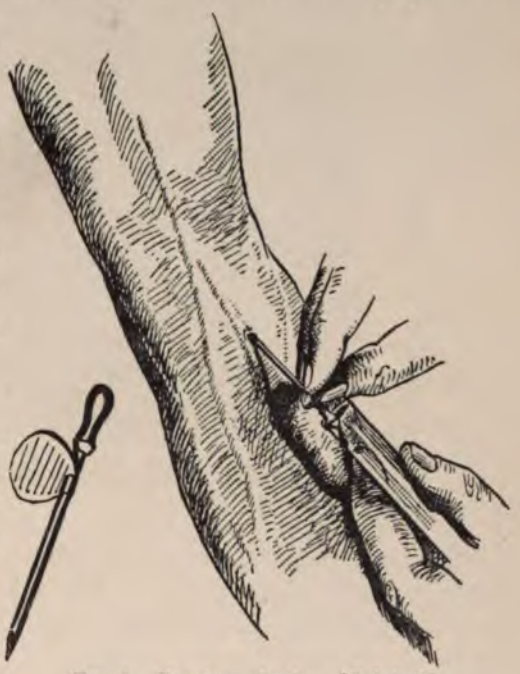

FIG. 6.—Puncture of vein. (*Original.*)

If the vein is prominent, the canula is thrust into the vein directly through the skin. Here the author has found it more convenient to point the canula upward, *i.e.*, in the direction of the blood stream.[1] In cases where the vein does not stand out it can be made to do so either by applying pressure with the finger upon its central part or placing a tight rubber bandage or rubber tube about the arm. This should not, however, be tight enough to obliterate the radial pulse. In very fat individuals, even these means do not suffice so that the vein must be dissected free and incised. After completion of the venesection the arm is elevated, slight pressure made upon the wound with sterile cotton and a bandage applied. If a small amount of blood is sufficient, and, as in most serological examinations absolute sterility is not essential, venesection can be replaced by the method of wet-cupping. For this procedure a scarifier and Bier cup are required. The technique is as follows (Fig. 7).

Wet-cupping.

[1] The editor has found that more blood is obtained by thrusting the canula into the vein in the reverse direction.

Some part of the skin of the back is thoroughly disinfected and a well-fitting Bier cup firmly applied. Suction arises and the skin assumes a dark bluish-red appearance. After half a minute the cup is removed, the scarifier applied and the cutting edges set free. The scarifier is then reapplied, but this time at right angles to the previous incisions and the edges again set free. Suction is again made by the Bier cup and the blood is thus forced out from the multiple incisions.

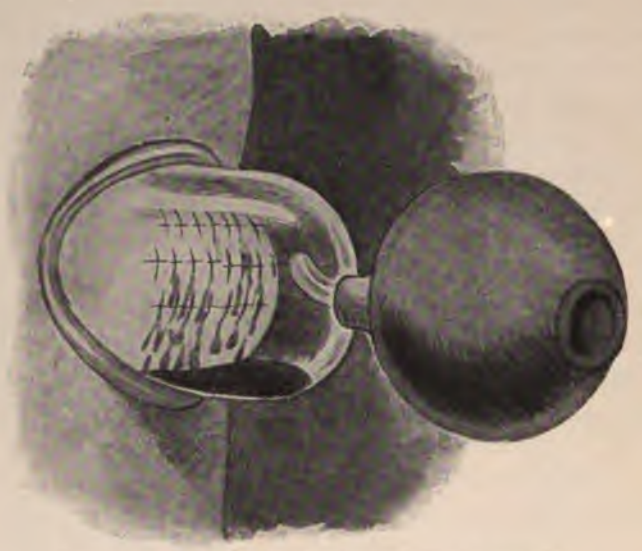

FIG. 7.—Obtaining blood by the wet cupping method.

The blood obtained by any of the above methods is collected into a sterile vessel (graduate, flask, test-tube) and allowed to coagulate. The clot is then separated from the sides of the vessel by a sterile glass rod or platinum needle, the vessel plugged with absorbent cotton and placed into the ice-chest. After 12 to 24 hours the serum begins to separate out from the clot. If the serum is required immediately, the blood is allowed to flow directly into centrifuge tubes, the clot separated from the sides and the tubes centrifugalized. With a well regulated centrifuge serum appears after several minutes.

Rules for the Use of Centrifuge.

There are several rules to be kept in mind when using a centrifuge.

1. The machine must be well oiled.
2. The counterweights must be absolutely of the same weight.
3. The centrifuge should never be suddenly stopped, but allowed to do so of its own accord.
4. In starting it, the motor should be gradually turned on.
5. If the centrifuge is slightly out of order it should not be used, but repaired at once, otherwise it may be ruined forever.
6. One should never centrifugalize with cotton plugs in the test-tubes. If the latter must be sealed, rubber stoppers should be used.

The color of a serum is greatly variable, depending mainly upon its hemoglobin or fat content. Blood taken at the height of the period of digestion shows a chylous serum. The serum of nursing women contains milk, that of icteric people contains bile. For most serological examinations these elements in the serum are inert, and do not interfere with the reading of the results. In precipitin reactions, however, the serum must be absolutely clear.

If serum is to be kept for a long time, there are several ways that it may be retained without losing its activity. The method chosen depends upon the serum substance, which is important.

As will be pointed out again, substances are either *thermostabile* or *thermolabile*. The preservation of thermostabile substances (agglutinins, amboceptors) is usually very simple. It is sufficient to place the clear serum which has separated from the clot, into a sterile test-tube plugged with absorbent cotton, and to put it into the ice chest away from the light. To reassure its perfect preservation one may add to it some phenol in such proportion that the carbolic should be present to the extent of 1/2 per cent. solution, *e.g.*, to nine c.c. of serum add 1 c.c. of a 5 per cent. phenol solution. The latter should be added drop by drop and agitated, so as to avoid the formation of precipitates. Another method, which the author employs almost exclusively for the preservation of amboceptor containing sera, consists simply in inactivating the sera at 56° C. for a half hour and then placing them into the ice chest. Inactivation has the advantage of stopping molecular changes which are produced by ferment actions of fresh serum. Furthermore, heating acts as a sterilizer for isolated air germs which may have found their way into the serum during the process of getting it. In this form, a serum can be kept in the ice box for several weeks without any material change. Occasionally one finds that a serum will undergo contamination in spite of inactivation, so that it follows, that if a serum is to be preserved for several months, it is advisable to seal it in a test tube. For this purpose a brown glass tube slightly drawn out at its upper end is employed (Fig. 8). The serum is placed into this sterilized tube and then the latter is sealed in the flame at its narrow part. Bacterial and organ extracts are well kept in this way. The best method of preservation consists in evaporating the serum to dryness in a *vacuum desicca or*. This procedure is rather complicated and can therefore be employed only in institutions.

FIG. 8. Tube used for preservation of fluids. *(Original.)*

A vacuum desiccator with heatable plates is used. The serum is poured in very thin layers into sterile flat dishes and allowed to dry out in the desiccator at a temperature of 30° C., later on at 35° C. in a vacuum of 3 cm. mercury. The dried serum forms a

yellowish-red horny mass which is scraped off from the dish and ground up in a mortar into a yellowish powder. The serum powder is then placed into a brown glass tube and sealed.

When this dried serum is to be used, the tip of the ampulla is broken off, and several drops of isotonic salt solution at a temperature of 30° are poured in, in just sufficient an amount to moisten the wall of the glass tube. By rolling the tube to and fro, one finds that the serum powder will easily stick to the moistened wall. The granules are allowed to swell up and after they have done so, enough isotonic salt solution is added to make up the original volume.

For the preservation of thermolabile substances, the method of freezing has been suggested. *Morgenroth* has devised for this purpose a simple and handy apparatus named *Frigo* which can be obtained from *Lautenschläger, Berlin*. Although for most tests this method of preservation has been employed with success, Neisser's clinic reports that sera preserved in the *Frigo* with the idea of retaining their complement did not give as accurate complement fixation experiments as did similar fresh sera.

Friedberger advises the addition of 8 per cent. salt solution for the preservation of the complement. When the serum is to be used it is diluted tenfold with distilled water, so that a 10 per cent. dilution of complement is obtained. By the addition of the salt, the resistance against the harmful effects of light, room, body temperature, and chemical substances like phenol is increased, but the thermolability of the complement remains the same. Drying of a serum in a desiccator is not to be advocated for the preservation of the complement as during such procedure a portion of the complement is lost. Once the serum is in its dried form, however, the remaining complement is retained and in addition, has become resistant against high heat.

Filtration of Bacteria.

It is important in many serological studies to be able to separate bacteria from their fluid media or suspension. This is accomplished either by centrifugalization or filtration. The first method does not completely free

FIG. 9.
Pukal-filter.

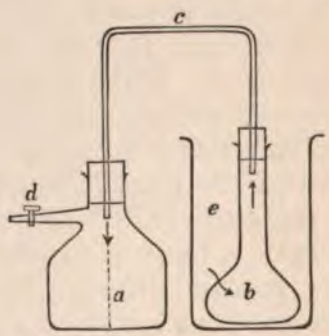

FIG. 10.—Filtration through a Pukal-filter.

the fluid of its bacteria, but if this is desired the method of filtration is essential. In this connection, however, one must bear in mind, that many albumenous, or albumen-like substances, few colloids and even some toxins, do not pass the filters and are therefore held back. Bacterial filtration is simplified by preliminary centrifugalization or passing the fluid through

filter-paper. Different porous materials have been used for bacterial filters; of which especially suitable are porcelain, infusorial earth and asbestos. The filtration apparatus consists of the respective filter and the receptacle which receives the filtrate. Filtration takes place by differences in pressure, where either the fluid is forced through by high pressure or sucked through by a vacuum formed in the receiving vessel. The following are some of the filters most commonly in use.

1. CHAMBERLAIN'S CYLINDER FILTER, F, used in the Pasteur Institute at Paris. The filter cylinder is made of infusorial earth and may be attached to any faucet.

2. PUKAL FILTER, made of burnt kaolin, is used especially for the filtration of large quantities of fluid. The filter *b* is placed into the beaker *e* containing the toxin and bacterial fluid. The former is then closed by a rubber stopper, perforated by a central opening through which runs a glass tube bent at right angles, and this in turn is connected with either an air or water pump for producing a vacuum inside of the filter. Between the filter and vacuum pump can be interposed a sterile jar a. (Figs. 9 and 10).

FIG. 11.—Reichel filter.

FIG. 12.—Lilliputian filter.

3. THE REICHEL FILTER (Fig. 11) consists of a glass receiver A, having a side neck *c* and at the bottom a tube-like outlet *d*. A porcelain filter B fits into the glass jar and rests upon the margin of the flask by means of a broad collar. The point of junction is made air tight by means of a rubber cap with a central opening, through which the cylinder can be filled. When in use *d* is shut off by a rubber tube with a pinch cock and *c* in which lodges a small piece of cotton is connected with a water pump that is instrumental in bringing about a vacuum. The function of *d* is to allow the removal of samples of the filtrate and finally to obtain the entire filtrate.

4. THE LILLIPUTIAN FILTER, candle-like in shape, and made of infusorial earth, is employed for the filtration of very small quantities. The filter is cemented upon a metal tube which is screwed, so that it is air tight, into a well-fitting glass cylinder open at the top. The tube is passed through a rubber cork which tightly closes an exhaust flask.

The fluid to be filtered is placed into the glass cylinder and sucked through into the flask by means of a vacuum which is here produced. For the purpose of collecting very small quantities a test-tube may be placed into the exhaust flask (Fig. 12).

Preparation of Dilutions and Measurement of Small Amounts of Bacteria.

All serological methods are to be considered on quantitative bases. In serum diagnosis as well as in the therapy, the amount of the serum employed is the deciding factor. Similarly, the number or amount of bacteria required either for the purposes of immunization or serological reactions is of extreme importance.

One cubic centimeter is the unit of measure for serum and all fluid material (Bouillon cultures, exudates, etc.). *If small quantities are required, it is best to dilute the fluid with* 0.85 *per cent. saline solution. The exact preparation of dilutions is one of the most essential technical procedures of all serum diagnosis.* Some general rules may be of help.

Never should amounts less then 0.1 c.c. be measured out directly. For beginners even 0.1 is best measured in the form of a dilution, as errors are apt to occur very easily.

2. The *decimal system* should be adhered to as much as possible.

3. The dilution should be made just before it is to be used, inasmuch as many substances retain their activity best, or only, in concentrated form.

The following is an example of correct forms of dilution:

Toxin.	Dilution of toxin, 1 : 10	Dilution of toxin, 1 : 100	Dilution of toxin, 1 : 1000
0.1 c.c. =	1 c.c.		
0.05 c.c. =	0.5 c.c.		
0.01 c.c. =	0.1 c.c.	= 1 c.c.	
0.005 c.c. =		0.5 c.c.	
0.001 c.c. =		0.1 c.c.	= 1.0 c.c.
0.0005 c.c. =			0.5 c.c.

The stock dilution of 1 : 10 is made by measuring off 1 c.c. of toxin an adding 9 c.c. of 0.85 per cent. of saline.

The dilution 1 : 100 can be made by taking 1 c.c. of toxin and adding 99 c.c. of saline. It is more practicable, however, to take 1 c.c. of the 1 : 10 stock dilution and add 9 c.c. of saline. If the dilution 1 : 10 is not present and only a small amount of the dilution 1 : 100 is desired, the latter is made by taking 0.1 toxin : 10.0 NaCl sol. Similarly 1 : 1000 = 0.1 : 100 = 1 c.cm. of the dilution (1 : 10) : 100 = 1 c.c. of the dilution (1 : 100) : 10.0.

The following table shows the details of various dilutions:

Dilution 1 : 10.	Dilution 1 : 100.	Dilution 1 : 1000.	Dilution 1 : 10,000.
1 c.c. + 9 c.c. NaCl sol.	1 c.c. + 99 c.c. NaCl sol.	1 c.c. + 999 NaCl	0.1 + 999.9 NaCl
= 0. c.c. + 0.9 c.c. NaCl solution.	= 0.1 c.c. + 9.9 c.c. NaCl solution.	= 0.1 + 99.9 NaCl	= 1 c.c. of dilution 1 : 1000
= 0.2 c.c. + 1.8 c.c. NaCl solution.	= 0.2 + 19.8 c.c. NaCl solution.	0.2 c.c. + 199.8 = 2 c.c. + 1998 c.c.	+ 9 c.c. of NaCl
= 0.3 c.c. + 2.7 c.c. NaCl solution.	= 0.3 + 29.7 c.c.	= 1 c.c. of dilution 1 : 100 + 9 c.c. NaCl = 0.1 c.c. dil. 1 : 10	= 0.1 c.c. of dilution 1 : 100 + 9.9 c.c. NaCl
= 3 c.cm. + 27 c.c. NaCl	= 1 c.c. of dil. 1 : 10 + 9 c.c. of NaCl. sol.	+ 9.9 c.c. NaCl = 0.1 c.c. of dil.	= 1 c.c. dil. 1 : 100 + 99 c.c. NaCl.
= 10 c.cm. + 90 c.c. NaCl.	= 2 c.c. of dil. 1 : 10 + 18 c.c. NaCl. sol., etc.	1 : 100 + 0.9 c.c. NaCl solution.	= 0.1 c.c. dil. 1 : 10. + 99.9 c.c. NaCl.

In carrying out these dilutions, it is best to measure off the small quantities 0.1 – 1 c.c. with a pipette; allow this to run into a well-graduated measuring glass and add enough saline to make the required dilutions. For example, if 30 c.c. of a dilution 1 : 100 is desired, .3 c.c. should be measured off with a pipette and allowed to flow into a 50 or 100 c.c. graduated cylinder and saline solution added up to 30.0 c.c.

It should always be one's aim to get along with small quantities of the substance to be diluted. If, for example, 8 to 10 c.c. of a toxin dilution 1 : 100 are required 0.1 c.c. of toxin + 9.9 c.c. of saline should be taken and not 1 c.c. of toxin and 99 c.c. of NaCl sol.

Before making any dilution one should always calculate the total amount of substance required; as for example in the following experiment:

1. Animal 0.1 c.c. Toxin subcutaneously
2. Animal 0.05 c.c. Toxin subcutaneously
3. Animal 0.01 c.c. Toxin subcutaneously
4. Animal 0.001 c.c. Toxin subcutaneously

Here, the total quantity of toxin necessary, is found by adding, to be 0.161 c.c. This represents the minimum amount. It is always advisable to make an allowance for some loss and at the same time bring up the amount to a round or even number. 0.2 c.c. of toxin would fulfill all these requirements. This amount is measured off by a pipette, placed into a graduated cylinder and saline added up to 2.0 c.c., making a dilution of 1 : 10. Then 0.2 of this dilution (1 : 10) is taken, placed into another graduate, and again diluted with saline up to 2.0 thus making a dilution of 1 : 100. The above problem therefore, of injecting the various animals, can be completed as follows:

1. Animal receives 1 c.c. of dilution 1 : 10
1. Animal receives 0.5 c.c. of dilution 1 : 10
3. Animal receives 1 c.c. of dilution 1 : 100
4. Animal receives 0.1 c.c. of dilution 1 : 100

The unit for measuring the amount of bacteria grown upon a solid medium is represented by a standard sized loop. This platinum loop takes up

about 2 mg. of bacterial substance. It is prepared as explained in Fig. 2. If smaller amounts of bacteria are used, dilutions must be made.

For instance, 1/4 of a loopful of bacteria is desired; 1 loopful is suspended in 1 c.c. of saline and 3 c.c. of saline added. As a result, 1 c.c. of this emulsion contains 1/4 of a loopful of bacteria. If 1/16 of a loopful is necessary 1 c.c. of the above dilution is added to 3 c.c. of saline, thus making 1 c.c. of this mixture contain 1/16 of a loopful of bacteria.

CHAPTER III.

Active Immunization.

IMMUNIZATION WITH LIVING AND DEAD VIRUS.

The Principle of Active Immunization.

Active immunization depends upon the principle, that an organism in overcoming a slight infection either naturally or artificially acquired, develops enough protective bodies to withstand a similar, severer, natural, or acquired infection. Moreover, it serves primarily the purpose of prophylaxis. In laboratories, active immunization of animals is also frequently undertaken with the view of obtaining sera for diagnostic and therapeutic indications.

In the manufacture of serum on a larger scale, the horse is the animal used almost exclusively. Occasionally cows, sheep, donkeys or mules are selected. In small laboratories usually rabbits, guinea-pigs, white mice, rats, and only occasionally goats or sheep are employed.

The process of immunization evokes a marked disturbance in the general health of the animals. For this reason they must be well kept in warm places, and well fed with nutritious food. As far as their power of producing antibodies is concerned, there are individual differences even among the same species of animals; thus if five horses are immunized against diphtheria, some will give much better curative sera than the others. In general, the younger animals are preferable.

Any substance which, when injected into an organism, can stimulate the production or formation of an antibody, has been conveniently termed "antigen." After the injection of such an antigen, special notice should be taken of the animal in reference to temperature, weight, the excitation of diarrhea or the occurrence of abscesses, infiltrates, edema or paralysis.

If an animal dies, a careful postmortem, and if possible, a bacteriological examination should be made. It should be the aim to ascertain if death was induced by the inoculated antigen, by contamination or secondary infection. One should always keep in mind the possibility of some of the animal epidemic diseases.

Animal Infections.

Epidemic diseases occurring in rabbits are:

1. Rabbit Sepsis.—Presents itself in the form of bronchopneumonia and marked nasal catarrh. It is very infectious. Sick animals should at once be isolated or killed and their cages thoroughly disinfected.

2. COCCIDIOSIS gives changes in the liver due to the settling of the coccidi ova forms. The parasites are easily recognized, with the microscope, as present in the pus.

Following labor, *guinea-pigs* are very susceptible to sepsis.

IN RATS, trypanosomiasis is of frequent existence, but is not pathogenic.

The Technique of Active Immunization.

The antigens are injected either *subcutaneously*, *intraperitoneally* or *intravenously*. Only on exceptional occasions is another entrance path chosen.

As regards the amount to be injected, one cannot, very well, give general rules. It is important to prevent severe reactions, although the question is still a disputed one, whether marked reactions tend to produce a better immunity. It is certain, however, that inoculations of antigens in such minute doses as to apparently give no reaction, can still lead to immunity and the production of antibodies.

Occasionally a single injection suffices for immunization. Repeated inoculations are usually necessary, especially so when a "highly valent" serum is desired, i.e., one containing a great number of antibodies or having high protective properties.

When repeated inoculations are undertaken, there are various methods of procedure.

1*a*. A small dose of antigen is injected. If a reaction sets in, one waits until this reaction has entirely subsided, then (not before the fifth day) the second injection—a somewhat larger dose—is given. After an interval of 5 to 8 days, a third injection of a still higher dosage is administered, and so on, again.

1*b*. The intervals are the same, but the amounts of antigen remain the same at each injection.

Both of these methods give excellent results and therefore are most frequently used.

2. For several successive days, a small or medium dose of antigen is injected. Each injection produces only a slight reaction.

This last scheme according to Fornet is especially suitable for the gaining of precipitation sera. As is evident, it has the advantage of gaining the immunity rapidly.

3. Inoculations are given at very long intervals (intermissions of four weeks or more). This method produces good sera, but has the disadvantage of requiring too long a time.

The methods of active immunization can also be divided according to the nature of the antigen.

1. Immunization with a living virus,
2. Immunization with a dead virus,
3. Immunization with bacterial extracts,
4. Immunization with bacterial toxins.

1. Immunization with a Living Virus.

This method of immunization simulates most closely the immunity attained spontaneously in overcoming an infection. Although this immunity is very strong, and lasts for a long period of time, its disadvantages lie in that it is attained with difficulty; frequently the dose of virus injected causes serious symptoms of infection. Various procedures have therefore been advocated to so diminish the toxicity of the immunizing agent that only immunization effects, and no toxic symptoms be obtained. This was attempted either by the reduction of the number of organisms employed, so that very minute doses were inoculated, or by the diminution of the infectious nature of these bacteria (virulence so called).

The first method, however, was not found applicable to all cases. The infectious nature of the different bacteria varies markedly. The same bacterium reacts differently with different animals. While some animals possess a natural immunity against certain bacteria, others exhibit a distinct susceptibility to the same micro-organisms. The conceptions therefore of *pathogenicity and virulence are purely of a relative nature.* In *talking of the pathogenicity of bacteria, one should always mention the class of animal for which these bacteria are pathogenic.*

Bail's Classification of Bacteria.

Bail has used this principle of pathogenicity in classifying bacteria. He mentions the following three classes:

a. Saprophytes.
b. Half or partial parasites.
c. Whole or pure parasites.

To the class of *saprophytes* belong all those bacteria which when injected even in larger doses do not produce any characteristic disease; these are also known as apathogenic—*e.g.*, hen cholera bacilli for human beings.

Classed as *half parasites* are those bacteria, according to Bail, the infectious nature of which depends upon the quantity of bacteria injected. While the injection of a rabbit with 1/1000 of a loopful of a typhoid culture will produce no evidences of disease, one-tenth of a loopful will result in slight increase in temperature, loss of appetite, and eventually a local redness at the site of the injection. One loopful may bring about the death of the animal. The manifestations are dependent entirely upon the number of bacteria injected. The smaller the number, the milder the symptoms, until one reaches the stage below which no disturbances at all are visible.

Pure parasites are those which have no subletal dose. Even the smallest amount, when injected, will produce death. As examples, the tubercle bacillus for guinea-pigs, and bacilli belonging to the group of Hemorrhagic Septicemia for rabbits. Of the last mentioned 1/10,000,000,000 of a loopful of some cultures kills a rabbit within twenty-four hours with the symptoms of a septicemia; in other words, the injection of 1 c.c. of a dilution of one loopful of culture in ten million liters of water suffices to kill the rabbit. Furthermore, the number of bacteria increases so greatly in the body of the rabbit that numerous bacteria can be demonstrated in every drop of blood and in all organs and body fluids.

The same organism is a saprophyte for the human being and a half parasite for the guinea-pig if injected subcutaneously and a complete parasite by intraperitoneal injection. *The conceptions therefore of complete or partial parasite as well as of saprophyte are only relative and are dependent upon the bacteria, the animal species, and the mode of injection.*

Example of Active Immunization with Living Bacilli.

It is now clear that immunization with living bacteria can only be undertaken if the latter belong to the class of half-parasites. Pure parasites are excluded from this method. As an example of such procedure can be given the immunization of a guinea-pig by intraperitoneal injections with living typhoid bacilli. Preliminary to this, the virulence of the typhoid culture must be ascertained.

a. Preliminary test to titrate the virulence of the typhoid culture.

1. Guinea-pig 1./I. 1909 1/20 loopful of typhoid culture intraperitoneal.
   2./I. active.
   8./I. alive.
2. Guinea-pig 1./I. 1909 1/10 loopful of typhoid culture intraperitoneal.
   2./I. active.
   8./I. alive.
3. Guinea-pig 1./I. 1909 1/8 loopful of typhoid culture intraperitoneal.
   2./I. slightly sick, does not eat.
   3./I. active.
   8./I. alive.
4. Guinea-pig 1./I. 1909 1/6 loopful of typhoid culture intraperitoneal.
   2./I. sick, does not eat, hair raised.
   3./I. still sick.
   4./I. more active.
   8./I. alive.
5. Guinea-pig 1./I. 1909 1/5 loopful of typhoid culture intraperitoneal.
   2./I. sick, does not eat, hair raised.
   3./I. very weak, when placed on side remains so.
   4./I. †
6. Guinea-pig 1./I. 1909 1/6 loopful of typhoid culture intraperitoneal.
   2./I. †

From this experiment it becomes evident that the letal dose of this particular strain of typhoid culture is 1/4 to 1/5 of a loopful for guinea-pigs by intraperitoneal injection. Immunization therefore must be started with a smaller dose —*e. g.*, 1/10 of a loopful.

b. Immunization.

1. Guinea-pig 8./I. 1909 1/10 loopful of typhoid culture intraperitoneal.
   16./I. 1/8 loopful of typhoid culture intraperitoneal.
   22./I. 1/4 loopful of typhoid culture intraperitoneal.
   Animal remains active and healthy.
   30./I. I loopful of typhoid culture intraperitoneal.
   5./II. 2 loopfuls of typhoid culture intraperitoneal.
   Animal remains active and healthy.
2. Guinea-pig 8./I. 1909 1/10 loopful of typhoid culture intraperitoneal.
   16./I. 1/4 loopful of typhoid culture intraperitoneal.
   Animal remains active and healthy.
3. Guinea-pig 8./I. 1909 1/10 loopful of typhoid culture intraperitoneal.
   16./I. 2 loopfuls of typhoid culture intraperitoneal.
   17./I. animal is sick and does not eat.
   18./I. animal is very weak.
   19./I. †

Control animals always die within twenty-four hours, as in previous experiment, on injection of 1/4 of a loopful.

From experiment with guinea-pig 1, it can be learned, that by gradual increase of the immunizing dose, a state of immunity is reached which can overcome an infection produced by a high multiple of the dosis letalis.

Experiment 2 and 3 prove that even a single preliminary injection suffices to prevent the death of an animal upon subsequent receipt of the lethal dose of the same bacteria; but that this single inoculation is not sufficient to prepare the organism against a very severe future infection. The attained immunity is therefore only relative, not absolute.

Analogously it is possible to immunize by subcutaneous and intravenous injections. The latter method is usually the one of choice when half parasites are employed, as the highest and quickest grade of immunity is thus reached. It carries with it, however, the greatest danger and frequently results in death to the animal.

The method of immunization with small doses of living, fully virulent bacteria, has thus far been made use of only in animals. *In man this experience has not been carried into effect. It is feared that the bacteria may increase very rapidly and give rise to severe disturbances.* The method has therefore been altered and *instead of using virulent material for immunization, only a weakly infectious or attenuated virus is employed.*

Vaccination against Small-pox.

Vaccine.

This is the best known example of active prophylactic immunization. To Jenner belongs the credit of having been the first one to apply this principle. Vaccination against small-pox consists in inoculation of an attenuated form of small-pox germs, the diminution in virulence being brought about by passage through the body of a calf, a less susceptible animal than man. The vesicles formed on the vaccinated person contain these attenuated germs. This lymph can be used for the inoculation of other individuals, as the germs do not regain their virulence by repassage through man.

Inasmuch as it is not within the scope of this book to go into the details of the preparation of the lymph or the technique of vaccination, a brief survey of the benefits of vaccination will amply suffice and this may be seen from the table hereunto appended.

The mortality from small-pox per 100,000 population was in the year

	1862–1876	1882–1896
in Prussia and Bavaria	51.6	0.7
in Austria	75.2	38.6
in Belgium	79.5	18.2
in England	25.3	2.9
in Sweden	26.9	0.5

This method of immunizing against a virulent virus by inoculating with an attenuated form of the same, is known as *Jennerization. Pasteur recognized that this method had general application and similarly used attenuated but*

still living cultures, "vaccins" so-called, to immunize against hen cholera, swine plague, and anthrax. The same principle underlies Pasteur's antirabic vaccination.

Antirabic Vaccination.

In all civilized countries their exist at present, special institutions, either directly under the city control or appointed by the city, where the Pasteur treatment for rabies is conducted. It is the duty of the general practitioner, on getting a suspicious case of rabies to advise his patient to undergo this special therapy and to send the rabid animal, its head or brain preserved in glycerin, to the institute as soon as possible for the purpose of ascertaining the presence of rabies. Up to the present the actual cause of hydrophobia is unknown. Most recently Negri has described parasites, known as Negri bodies, in the large nerve cells of the cerebral cortex, cerebellum, etc. Pasteur found, that rabies can be transmitted to dogs by injecting them subdurally with the brain substance of rabid animals. This ordinary virus containing material is known as Street Virus.

Principle of Rabies Treatment.

The incubation period of rabies is very long. It varies from about three weeks, to [possibly], some years. By passing the virus through monkeys, the incubation period is considerably increased. After the successive passage through five or six animals, the virus becomes so weakened that infection is almost impossible. Reversely, increase of the virulence may be affected by passing the virus through a successive number of rabbits which are very sensitive to the disease. After passage through a large number of such animals, the incubation period is gradually shortened from about three weeks or a little less to a constant period of six or seven days. Further diminution in the period of incubation was impossible and therefore Pasteur called this "*Virus fixe.*" His first experiments in immunization were made by passing the weakened monkey virus through rabbits and then treating dogs with the spinal cords of the latter.

Later on, Pasteur discovered that instead of passing the virus through monkeys, he could *diminish its virulence by drying the spinal cords* derived from rabid animals, for varying periods of time. In this way he could prepare an entire series of graduated strengths. The material used for this drying was not the street virus, but that obtained by successive passage through rabbits or "virus fixe" which possessed very constant immunizing and infectious properties. By drying the "virus fixe" over caustic potash at a temperature of 23° to 25° C. for five days, its regular incubation period of 7 days was very much prolonged. Increase in the length of drying caused the entire loss of virulence in the spinal cord.

Pasteur immunized dogs as follows: He began with the injection of a virulent spinal cord which had been dried for thirteen days and every following day injected

subcutaneously some fresher spinal cord, *i.e.* (dried for a lesser period of time), until finally he used virus dried only for one day. The animals thus treated were immune against the bites of rabid dogs as well as subdural, subcutaneous, and intravenous infection with "virus fixe" and street virus. This procedure was strongly recommended by Pasteur, who brilliantly contributed the observation, that if an animal was infected but did not as yet show symptoms, these could be prevented by a similar modus operandi, as above mentioned.

In man, the inoculation is carried out on the same principle. The fact that the incubation period of hydrophobia is very long, makes the prophylactic inoculations of greater service. Only rarely is this period less than six weeks, usually considerably longer—up to 584 days, entirely dependent upon the virulence of the virus and the point of infection.

Technique of Antirabic Vaccination in Man.

The actual vaccine consists of 1 c.c. (2-3 mm. length) of the substance of the spinal cord of a rabbit which has been killed by inoculation with the fixed virus, rubbed up into a fine emulsion with 5 c.c. of sterile 0.85 NaCl solution. About 1 to 3 c.c. of the resulting fluid are injected subcutaneously into the skin of the abdomen. A cord dried for fourteen days is used for the first injection, emulsions of less attenuated virus are used on succeeding occasions until finally a portion of a spinal cord dried for only three or four days is employed. Pasteur's schemes of the actual doses can thus be drawn up.

a. For infections at points distant from the central nervous system (*mild infections*).

Day of injection	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Number of days cord was dried.	14 + 13	12 + 11	10 + 9	8 + 7	6 + 6	5	5	4	3	5	5	4	4	3	3
Amount injected..	3.0	3.0	3.0	3.0	2.0	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0

b. For head wounds (*severer infections*).

Day of injection..	1		2		3	4	5	6	7	8	9
Number of days cord was dried.	14 + 13 In A. M.	12 + 11 In P. M.	10 + 9 In A. M.	8 + 7 In P. M.	6+6	5	5	4	3	4	3
Amount injected.	3.0 A. M.	3.0 P. M.	3.0 A. M.	3.0 P. M.	2 C.C. A. M. 2 C.C. P. M.	2.	2.	2.	1.	2.	1.

Day of injection..	10	11	12	13	14	15	16	17	18	19	20	21
Number of days cord was dried.	5	5	4	4	3	3	5	4	3	5	4	3
Amount injected..	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.	2.

The drawback to this classical method of Pasteur, consists in using the virulent material rather late in the course of the inoculations. A more energetic treatment has therefore been advised. There is no added danger in doing this because *the virus fixe in contrast to the street virus is not at all or only slightly infectious for man.*

Högyes in Buda Pesth uses the virus fixe right from the start. He begins with marked dilutions (1/10,000) and gradually increases them to 1/100. The theory underlying this procedure is, that the usual method of attenuation by drying alters the quantity of the virus but not its quality; hence the same result may be obtained by simple dilution.

Ferran successfully employs the virulent virus in large doses right from the onset of the treatment. Especially in very severe infections, as in bites from wolves, is this procedure justifiable.

The exact arrangement of doses varies a little at different institutions. In Berlin, it is considered that the virulence of the dried cord is lost on about the eighth day instead of the fourteenth. Hence in the hydrophobia department of the Berlin Institute for Infectious Diseases, the authorities have adopted the following scheme, which stands midway between Pasteur's classical method and the extreme procedure of Ferran.

Scheme for treatment of mild infections:

Day of injection....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Number of days cord was dried.	8–7–6	5–4	4–3	5	4	3	3	2	2	5	5	4	4	3	3	2	2	4	3	2	2
Amount injected in cubic centimeters of an emulsion 1 c.c. of cord in 5 c.c. of sterile bouillon.	0.5 of each	1.5 of each	2.0 1.0	3.0	3.	1.5	2	1	1	2	2	2	2	2	2	1.5	1.5	2	2	1.5	2

Scheme for treatment of severe infections.

Day of injection.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Age of cord.....	8–7–6	4–3	5–4	3	3	2	2	1	5	4	4	3	3	2	2	4	3	2	2	3	2
Amount.....	1.5	1.5	1.5	2	2	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2

In severe injuries the entire treatment is repeated after one month's interval.

There is at present no doubt whatsoever as to the value of these antirabic vaccinations. While the mortality, compiled from a great number of statistics, of the untreated cases, of those infected or exposed to infection is 15 to 16 per cent., the death rate of those treated at the Berlin Institute during 1898 to 1901 was 0.55 per cent. Similar figures are given by the other institutions.

Vaccination Against Tuberculosis.

Attempts have been made to employ this principle of virus attenuation for other infections. Behring and Koch tried immunization against bovine tuberculosis by inoculation with living human tubercle bacilli. The material used for the above inocula ions can be bought under the name of Bovovaccine (v. Behring) and Tauruman (Koch).

Tauruman is prepared by the Höchst Farbwerke and is put up in sealed glass tubes which contain 0.02 to 0.04 gm. of living tubercle bacilli suspended in 10 c.c. of normal saline solution. This Tauruman is previously examined in Ehrlich's Institute and note is taken of its purity, quantity of bacteria, virulence against guinea-pigs and avirulence against rabbits (characteristics of the human type of tubercle bacilli).

To this class of experimental work belong also the attempts of Friedmann to immunize against human tuberculosis by the use of the tubercle bacilli of cold blooded animals, and those of Wassermann, Ostertag and the author, to inoculate against hog cholera with living cultures of mouse typhoid.

Other Methods of Vaccine Preparation.

Besides the preceding way of virus attenuation by passage through animals, there are other methods employed for the diminution of the toxicity of the virus. Growing the bacteria at too high a temperature, or exposing bacterial emulsions to light, disinfectants or moderate heating, accomplishes the same purpose.

The mixture of bacteria with their specific serum (*i.e.*, serum obtained from animals that have been inoculated with these bacteria), also diminishes the strength of the inoculated bacteria. Such bacteria are designated by Bordet as "sensitized" bacteria. By allowing this mixture to remain for some time, the bacteria attach their specific antibodies so that after centrifugalization the added specific serum, now devoid of its specific antibodies is removed, and the sensitized bacteria can be used as vaccines. Inoculations of the latter rarely produce any infiltration. The same object

can also be accomplished by injecting bacteria and at the same time also their specific serum. This is technically simple and is known as the "*Simultaneous Method.*" It has shown itself to be of great value in Lorenze's prophylactic inoculations against swine erysipelas.

2. **Immunization with Dead Bacteria.**—Immunization with dead bacteria was first performed by Toussaint, Salmon and Smith, and Chamberland and Roux.

This method is to be distinctly separated from those already discussed. Bail claims that the immunization with living bacteria as well as by aggressins (to be mentioned later) is an immunization against the infectious disease; while the immunization with dead bacteria is an immunization against the bacterial bodies. While this holds true for some bacteria, it is, to say the least, questionable whether it can be considered as a general rule.

Whenever a real immunity is desired—that is, protection against *disease*, a vaccine either in the form of living or attenuated bacteria should be given the preference. Up to a certain degree the extracts of living bacteria, and the natural and artificial aggressins can be similarly employed. If, however, no real immunity, but just a serum containing a great number of antibodies is wanted, as in serum diagnosis, for agglutination, bacteriolysis, complement fixat on, etc., then immunization by dead bacteria is just as, if not more so, efficient.

Recently, the question has been raised whether the antibodies produced by immunization with heated antigens are identical with those obtained with unheated antigens. The experiments of Obermeyer and Pick which will be referred to under proteid immunization, seem to prove that they are not alike. For laboratory work it is advisable to use living cultures only in cases of absolute necessity.

Death of Bacteria by Heat.

In heating bacteria to destroy their virulence and thus be suitable for inoculation, we must be very careful not to raise the temperature to such a degree where not only the toxicity but also the immunization power is destroyed. It is best to employ the minimum amount of heat which will kill the respective bacteria. For most of these as Typhoid, Paratyphoid, Colon, and Dysentery bacilli, Cholera Vibrios, Meningo-, Staphylo-, Strepto- and Pneumococcis, one hour at 60° C. is sufficient.

The bacteria are grown upon agar cultures and the required amount is removed and suspended in sterile physiological salt solution or bouillon. This suspension is then placed into a hot water bath or thermostat regulated at 60°, for one hour.

If the bacteria employed are highly infectious, one must be sure that all bacteria have been killed. This must especially be noted when giving *prophyl*actic inoculations in man. Several drops of the emulsion are there-

fore subplanted on agar tubes and incubated for a day or two. If a growth appears, the emulsion is to be reheated; if not it can be considered sterile.

The mode of immunization is the same as has been described for the living bacteria. In general the dosage to be used may be larger.

Small doses are injected at first, followed later on by increasing quantities at intervals of five to eight days. *e.g.*

Intravenous inoculation of a rabbit with dead typhoid bacilli.

Result.—Protection against living virulent bacteria, appearance of agglutinins, bacteriolysins, bacteriotropins and complement binding substances in the serum.

1./I. 1909.	Rabbit No. I.	1 loopful of a typhoid agar slant culture killed at 60° and injected intravenously.
6./I.		4 loopfuls of typhoid culture killed at 60° and injected intravenously.
12./I.		1 culture of typhoid killed at 60° and injected intravenously.
20./I.		Infection with 1 culture of the living typhoid bacilli injected intravenously. Animal remains alive.
	Rabbit No. 2.	Control.
20./I.		Infection: 1/4 loopful of living typhoid bacteria intravenously.
22./I.		† (death).

The use of killed typhoid bacteria for prophylactic immunization has recently been widely adopted. This has been stimulated to a great degree by the successful experiments of Wright, and Pfeiffer and Kolle.

Wright's Method of Prophylact c Typhoid Inoculation.

The vaccine or ginally employed by Wright for these inoculations consisted of highly virulent cultures of Bacillus Tpyhosus grown in broth for twenty-four to forty-eight hours (sometimes even for four weeks), and sterilized by heating at 60° C. The vaccine was then standardized, *i.e.*, the strength of the vaccine was fixed in accordance with another of known strength, the dosage of which had been gauged by inoculations in man.

The early form of standardization consisted in determining the toxicity of the virus. Guinea-pigs weigh ng 250 to 300 gm. were inoculated subcutaneously with 0.5, 0.75, 1.0 and 1.5 c.c. of the vaccine respectively. Death to some of the animals would come in twelve hours to three days. The amount required to kill a guinea-pig weighing 100 grammes or rather the proportional fraction of the dose which proved fatal to the one of 250 to 300 gm. was taken as the standard dose for injection in man. Wright subsequently found that better results were obtained, if the vaccine was prepared from twenty-four hour cultures grown upon the surface of agar, and after emulsification, standardized so as to contain 1,000 millions of typhoid bacilli in every cubic centimeter. This method of standardization, the details of which will be given in the chapter on Opsonins, is effected by

counting the number of bacteria under the microscope. At the first inoculation, the patient received 750 to 1000 million of these dead bacteria and at the second, eleven days later double the first dose injected.

Local and general reactions follow the inoculations. Thus local redness and swelling of the skin, lymphangitis and enlargement of the neighboring glands are the usual consequences. The inflammation can at times be severe enough to simulate erysipelas. The general symptoms, on the other hand, may consist of a general feeling of illness, headache, a little fever, and occasionally nausea, not infrequently accompanied by vomiting. These signs of indisposition, however, pass off rapidly without leaving any permanent ill effects. Six to eleven days after the injection, an increase in the number of agglutinating, bacteriolytic and bacteriotropic bodies can be demonstrated in the blood of the inoculated individual.

As to the effects of these inoculations opinion is somewhat divided. According to Wright's statistics infections have been diminished by about one-half, and in single series to one-sixth or even one-twenty-eighth of the former, or control number. The mortality too is much lower. Out of 1758 individuals who had been vaccinated, only 142 or 8 per cent. died; out of 10,980 who had not been, 1800 or 16.6 per cent. met death. From numbers such as these, Wright has come to the opinions he holds, and he moreover believes that the period of time during which prophylactic immunity can be maintained is from two to three years.

Pfeiffer-Kolle's Experiments.

Pfeiffer and Kolle prepare their vaccine by growing typhoid bacilli on agar cultures and suspending a twenty-four hours growth in physiological NaCl solution. The normal platinum loop is the unit of standardization. A full grown agar culture is considered as 10 normal loops and as such it is diluted in 4.5 c.c. of saline. This emulsion is placed into a thermostat at 60° for two hours and then tested for its sterility. Sufficient 5 per cent. phenol solution is next added to the suspension to make up the contents to a 0.5 per cent. carbolic solution, and the final emulsion is again heated at 60° C. for thirty minutes. One c.c. of the vaccine is thus equivalent to two normal loops of culture. The amounts of vaccine to be injected have not yet been definitely decided upon. The best dosage so far is the following:

For the 1st injection: 0.3 c.c. of the vaccine.
For the 2d injection: 0.8 c.c. of the vaccine.
For the 3d injection: 1.0 c.c. of the vaccine.

The injection is made subcutaneously between the breast and clavicle.

The local and general reactions are the same as those observed with Wright's method. As a result of the injection only increased agglutinins

and bacteriolysins, have been found in the blood serum. Bacteriotropins have not as yet been examined for.

The effects of these inoculations seem to be very good. Protection is prolonged according to the increase in the number of injections, and if inoculated individuals do become infected, they run a very much milder course of the disease.

The following statistics as given by Kuhn indicate the results:

	Inoculated.	*Non-Inoculated.*
Very slightly ill	186 (50.13 per cent.)	331 (36.55 per cent.)
Moderately ill	96 (25.88 per cent.)	225 (24.85 per cent.)
Badly ill	65 (17.52 per cent.)	234 (25.80 per cent.)
Deaths	24 (6.47 per cent.)	116 (12.80 per cent.)
	371 (100 per cent.)	906 (100 per cent.)

The prophylactic immunity according to Kuhn lasts one year. Kolle has undertaken similar experiments against cholorea.

CHAPTER IV.

Active Immunization.

Immunization with Bacterial Extracts.—Aggressin Experiments.

The marked infectious nature of the organisms belonging to the class of "pure parasites," makes it very difficult to produce an immunity against them. They possess no sublethal dose in their living state, and if used when dead, will produce no prophylactic immunity. Pasteur therefore, by artificial attenuation of these living virulent bacteria, had succeeded, in part, to obtain vaccines of several of them. The methods, however, that he employed were totally impracticable, for not infrequently in the use of the vaccine, the disease which it was the object to prevent, was instigated. It was therefore a distinct and important triumph when Bail and Weil showed that immunity against these parasites could be attained by using as vaccine antigen, the so-called *"aggressins;" i.e.*, exudates from animals that had been infected with the respective bacteria.

Bail's explanation of the aggressin-immunization method is entirely theoretical. He believes that during an infection, the bacteria secrete certain agents which counteract or entirely destroy the infected organism's protective powers, especially phagocytosis. These bodies he called aggressins and they were distinguished by the fact that they were formed by living bacteria, and only in the living body. According to Bail, the pathogenicity of bacteria depends upon their power to produce these aggressins. If this theory be correct, it should be possible to demonstrate aggressins, especially in infections where the protective power of the organism is almost nil, as for example an infection produced by the bacteria belonging to the group of hemorrhagic septicemia. Unfortunately, in actual practice this is not so.

The following experiment, however, gives an idea of the true nature of these aggressins and how they are obtained.

At first, an infecting agent—the bacillus of swine pest, may be chosen. This micro-organism belongs to the same class as chicken cholera and fowl plague, and is distantly related to the human pest. For rabbits, this bacillus is a pure parasite, for guinea-pigs, by subcutaneous inoculation, a half parasite.

The Obtention of Aggressins.

One drop of a twenty-four-hour broth culture of this swine pest bacillus, in 5 c.c. bouillon, is injected intrapleurally into a rabbit in the following manner.

A small incision is made in one of the intercostal spaces on the side of the chest, and through this wound a long canula is introduced into the pleural cavity. Following the injection, the animal as a rule, rapidly succumbs to the infective organism. On autopsy, the pleural cavity is found to contain an exudate of a reddish-brown color (hemorrhagic) on the side where the inoculation was given, and of yellow serous on the other side. This bloody exudate measuring about 15 c.c. is removed with a sterile pipette, placed into a sterile centrifuge tube to which is added 1.5 c.c. of 5 per cent. carbolic acid drop by drop (making the entire solution a 1–2 per cent. carbolic acid dilution), agitated continually in order to prevent precipitation, and followed by centrifugalization at a very high speed for many hours until it becomes very clear. The upper clear part which is now free of bacteria, or very nearly so, is drawn off by a pipette and heated for three hours at 44° C. Its sterility is then tested and if no growth appears after forty-eight hours, it is considered sterile.

First Fundamental Aggressin Test.

(*Its power of increasing severity of infections.*)

No.	Animal.	Date.	Amount of Infective Material.	Aggressins.	Result.
1	Guinea-pig.	6/IV '05.	1/100 loopful of swine pest subcutaneously.	.....	Remains alive.
2	Guinea-pig.	6/IV '05.	1/100 loopful of swine pest subcutaneously.	+1.5 c.c. of aggressins subcutaneously.	† on third day.
3	Guinea-pig.	6/IV '05.	.....	+1.5 c.c. of aggressins subcutaneously.	Remains alive.
4	Guinea-pig.	6/IV '05.	1/100 loopful of swine pest subcutaneously.	+3 c.cm. subcutaneously.	† on second day.
5	Guinea-pig.	6/IV '05.	.....	3 c.cm. subcutaneously.	Remains alive.
6	Guinea-pig.	6/IV '05.	1/1000 loopful subcutaneously.	+2 c.cm. subcutaneously.	On 7/IV very ill; on 8/IV very ill; 9/IV very ill; 10/IV very thin; 11/IV begins to pick up slowly and remains alive. Marked infiltration around point of injection.

It can be deduced from this experiment that 1/100 of a loopful of swine pest, which represents 1/10 of a fatal dose for a guinea-pig by subcutaneous injection, can be converted into an acutely fatal dose by injecting the aggressin simultaneously or a half hour before the experiment.

The aggressin itself is only *slightly toxic*, and the quantity injected is well borne by the guinea-pig. Its power of increasing the virulence of the infective material varies directly with its quantity, *i.e.*, the greater the dose of aggressin, the more rapidly is death occasioned. If, however, only small doses of the culture are given, and in addition to this, the aggressin is injected, the animal does not die, but becomes exceedingly ill, thus indicating the effect of aggressins. In this connection it might be well to add that the aggressin may be given twenty-four hours previous to the time of infection.

On microscopical examination of the aggressin exudate, of only very few cells, but a great number of bacteria are present. The bacteria here have increased during the short time after the infection to a far greater extent than they would have done in an artificial medium. The body, continually combatting against their increasing toxicity, finds itself powerless when its limited fighting capacity, decreasing in proportion to the rise in strength of the hostile micro-organisms, is expended, and ultimately succumbs to the infection. During the struggle between the protective forces of the organism and the invading bacteria, many of the latter are destroyed and these disintegrated bacteria are found within the exudate. From this fact Wassermann and Citron have formed the conclusion that the aggressins are not as Bail claimed, secretory products of live bacteria produced during the conflict between the bacteria and the body organism, but rather the products of broken down bacteria. Therefore, Bail's supposition that aggressins are only obtained in the living body is erroneous and can be shown to be so by the fact that aggressins may be reproduced whenever the essential requirements can be had, and these are:

1. Large numbers of bacteria.
2. Non-poisonous agents which can disintegrate these bacteria.

Aggressins thus obtained are known according to Wassermann and Citron, as "artificial" in contrast to Bail's "natural" ones.

Wassermann and Citron Method of Obtaining Artificial Aggressins.

Cultures are grown in mass on Kolle's flask plates. A Kolle's agar plate is equivalent to twelve agar slants. For the inoculation of these flasks a long platinum loop is needed which transfers some of the culture to the plate. The transferred material is then spread over the entire surface of the flask by a large triangular platinum loop. The latter is made by inserting into a holder both ends of a not too thin platinum wire, about 20 cm. *in length* which is then shaped into a triangular form. While still red hot,

this triangular loop should be introduced into the flask and allowed to cool there. Before the culture is spread, it is advisable to bend the entire loop to a slight angle by pressing it against the upper uncovered wall of the flask, thereby preventing the hot end of the loop holder from coming in contact with the agar surface. It is best also to test the platinum loop upon the surface of the agar in order to ascertain whether it is still too hot.

After twenty-four hours of incubation there is usually a pronounced growth upon the plates. This culture is then washed off either by serum or distilled water ("serous" or "aqueous aggressin"). The former may be obtained fresh from a rabbit. Usually 10 to 12 c.c. of fluid per flask is required; 3 or 4c.c. are first poured upon the culture growth and the mass scraped gently but quickly with the triangular loop. Then the remainder of the fluid 7 to 8 c.c. is poured in to release the still adherent bacteria. The turbid milky emulsion is collected either in a small dark glass Erlenmeyer flask or a brown bottle. This is then placed into a proper apparatus and shaken for one to two days at room temperature. Enough carbolic acid is added to make a 1/2 per cent. phenol solution, and the emulsion is centrifugalized and sterilized in the same manner as has been described for the natural aggressins.

The tendency of aggressins towards increasing virulence ("infektions beförderung") is the same whether these aggressins are artificial or natural.

From the following experiment it can be seen that the bacteria contain some substance which is easily soluble in the body fluids and in distilled water, and which has a proclivity toward increasing the infectious nature of their respective bacteria when injected simultaneously with them. In small doses, this substance is not poisonous, in large doses it may be, but is not necessarily so. There is no definite relation between the poisonous *qualities of the aggressin and its power to increase the virulence of an infection.* This disproves the assumption of some authors that the action of the aggressins is dependent upon the toxicity of the endotoxins.

Experiment.

No.	Animal.	Date.	Amount of infective material.	Serous Aggressin.	Watery Aggressin.	Results.
1	Guinea-pig.	29/V '05.	1/200 loop of swine pest subcutaneously.	.....	.....	Remains alive.
2	Guinea-pig.	29/V '05.	1/200 loop of swine pest subcutaneously.	+2.5 c.c. subcutaneously.	.....	† after twenty-four hours
3	Guinea-pig.	29/V '05.	.....	+2.5 c.c. subcutaneously.	.....	Remains alive.
4	Guinea-pig.	2/VI '05.	1/200 loop of swine pest subcutaneously.	.....	2 c.c. subcutaneously.	† after three days.
5	Guinea-pig.	2/VI '05.	1/200 loop of swine pest subcutaneously.	.....	3 c.c. subcutaneously.	† after three days.
6	Guinea-pig.	2/VI '05.	.....	.....	3 c.c. subcutaneously.	Remains alive.
7	Guinea-pig.	2/VI '05.	.....	.....	4.5 c.c. subcutaneously.	† in twenty-four hours.
8	Guinea-pig.	2/VI '05.	1/200 loop of swine pest.	.....	.....	Remains alive.

Second Fundamental Aggressin Test.

(*Its Property of Active Immunization.*)

Bail and his pupils believe that when bacteria invade a normal organism, it is the aggressin power of these bacteria which determines whether or not, by their multiplication disease will set in. If it does, the infection continues until the "aggressive" nature of the bacteria is curbed. As there are some bacteria which on injection do not produce any disease, Bail attributes this phenomenon of immunity to the missing "aggressive" action of the respective bacteria. It is not merely the presence of bacteria which is the criterion for the existence of disease; as long as they are void of their "aggressive" property, they have actually become saprophytes.

Accordingly, Bail believes that the bactericidal immunity is no true immunity because it can be obtained by injection of dead micro-organisms or by live bacteria in such minute doses that no specific symptoms are produced, *i.e.*, no aggressins are produced within the body. "If the immunity lacks the "anti-aggressive" component, which alone governs the existence of disease, one gains only an *apparent immunity* against the exciting factor of the disease, but not against the *disease itself.*"

Bail places the utmost stress upon the difference *between an immunity directed against the exciting agent* of the disease (bactericidal immunity) and that against the disease itself (anti-aggressive immunity).

Immunization against the disease is only possible if the *aggressin* reaches the body of the animal to be immunized. This is possible either by employing Pasteur's method of vaccine inoculation, *i.e.*, the injection of bacteria, the "aggressive" nature of which has been weakened but not destroyed, or by direct inoculations of aggressins. The latter is by far the simpler and more reliable mode of procedure, being productive of a true immunity.

Immunity Against Pure Parasite.

Nowhere does this problem appear of such extreme importance as where immunity against a pure parasite is contemplated as in the case of swine pest and chicken cholera. While it is exceedingly difficult, in fact almost impossible to immunize against these bacteria either with dead or living germs or vaccines, this task is readily accomplished by the injection of non-poisonous aggressins, inasmuch as they are well tolerated. In addition these bacteria are of help in definitely deciding whether or not an aggressin immunity is at all possible.

Weil, a co-worker of Bail's, has carried out these experiments for chicken cholera, while the author has done the same for swine pest.

Example of Active Immunization with Natural Aggressins.

a. Slow Immunization.

Rabbit I.

6./IV. 1905	1st injection: 1.0 c.cm. natural swine pest aggressin intraperitoneally.
17./IV.	2d injection: 1.0 c.cm. natural swine pest aggressin intraperitoneally.
25./IV.	3d injection: 1.0 c.cm. natural swine pest aggressin intraperitoneally.
1./V.	4th injection: 2.0 c.cm. natural swine pest aggressin subcutaneously.
12./V.	5th injection: 2.0 c.cm. natural swine pest aggressin subcutaneously.
16./VI.	1st infection; with 1/100 loopful of swine pest culture intravenously.
17./VI.	Perfectly well.
8./VII.	2d infection; with 1 loopful of swine pest culture intravenously.
15./VII.	Perfectly well.
22./IX.	3d infection; with 1 loopful of swine pest culture intravenously.
3./X.	Perfectly well.

Rabbit I.		*Controls.*	Rabbit II.
16./VI. 1905	1/100,000 loopful of swine pest culture intravenously.	8./VII. 1905	1/10,000 loopful of swine pest culture subcutaneously.
17./VI.	† found dead.	9./VII.	† found dead.

b. Rapid Immunization.

Rabbit II.

8./IV. 1905 Injection of 4 c.c. of natural swine pest aggressin subcutaneously.
10./IV. Animal somewhat depressed.
13./IV. Perfectly active.
26./IV. 1st infection: 1/10 loopful of swine pest culture subcutaneously.
16./VI. 2d infection: 1 loopful of swine pest culture subcutaneously.
Animal remained active.

Controls.

Rabbit III.

26./IV. 1905 1/10,000 loopful of swine pest culture subcutaneously.
27./IV. †.

These experiments prove conclusively that by the method described above, it is possible to attain a high grade of immunity. In this connection, however, it is very important to adhere to what Bail pointed out, namely, *that a long period should elapse between the last inoculation with the aggressin and the first infection; the reason for that being, that during the period of immunization, and following it for a longer duration of time, there is a condition of hypersusceptibility to infection.*

Example of Active Immunization with Artificial Aggressins.

Rabbit 1.

3./VI. 1905 1st injection: 4 c.cm. of watery extract of swine pest bacilli subcutaneously.
14./VI. 2d injection: 2 c.cm. of watery extract of swine pest bacilli subcutaneously.
25./VI. Removal of some blood.
4./VII. 3d injection; 3 c.cm. of watery extract of swine pest bacilli subcutaneously.
21 /VII. Infection: 1/10 loopful of swine pest culture subcutaneously.
3./X. Animal alive and healthy.

Rabbit 2.

19./VI. 1905 1st injection: 2.5 c.c. of serous extract of swine pest bacilli subcutaneously.
9./VII. 2d injection: 2.0 c.c. of serous extract of swine pest bacilli subcutaneously.
12./VII. 3d injection: 4.0 c.c. of serous extract of swine pest bacilli subcutaneously.
24./VII. 1st infection: 1/10 loopful of swine pest culture subcutaneously.
22./IX. 2d infection: 1 loopful of swine pest culture intravenously.
Animal remains perfectly well.

Rabbit 3.

16./VI. 1905 Injection: 2.5 c.c. of watery extract of swine pest bacilli subcutaneously.
4./VII. Infection: 1/100 loopful of swine pest culture subcutaneously.
15./VII. Small local infiltrate. Very active.
3./X. Animal alive and perfectly well.

Control animals inoculated on the days of infection died within twenty-four hours after inoculations of 1/100,000 loopful of culture intravenously, 1/10,000 loopful subcutaneously.

It is evident from the above, that an immunity against pure parasites can be obtained just as well *by one or several injections of extracts of living bac-*

teria, as by injections of natural aggressins. As the possibility of the production of aggressins by a struggle between the bacteria and distilled water can be excluded, it can be taken without further explanation that in the development of those substances which have a tendency to increase the virulence of bacteria, or which can be used to produce an immunity, the bacteria play a passive rôle, in that they are only extracted by the dissolving agent. The difference between the anti-bacterial and anti-aggressin immunity is therefore not a qualitative one, as in both instances it is the substances that are set free from the bacteria which stimulate the formation of antibodies. When *living virulent bacteria are injected for the purposes of immunization*, they increase so rapidly that a proper dosage is impossible and the animals frequently die before enough antibodies are liberated. In addition antibodies are also generated against the capsule of the bacteria (bacteriolysins).

The only difference between immunization with *morphologically well preserved* but dead bacteria and that with aggressins is that within the latter the bacterial substances which tend to bring about the immunity have not been altered by previous heating, but exist in their natural easily absorbable form. Moreover, by using the extracts one does away with certain toxic substances which are found within the bacterial capsules, and which are rather toxic to subcutaneous tissue, producing necrosis and marasmus.

The Third Fundamental Aggressin Experiment.

Here, it is demonstrated that the serum of animals immunized by aggressins either artificial or natural, contain antibodies which (1) can neutralize that property of aggressins whereby they increase the virulence of bacteria; (2) produce a passive immunity against infection with living bacteria.

As for the biological structure of these antibodies, or anti-aggressins as they may be called, it may be said that they belong to the class of amboceptors, shown by the complement fixation methods.

The practical employment of aggressins as a method of immunization offers distinct advantages, namely:

1. Absence of any possible dangerous effects.
2. Absence of or only very slight local and general reactions.
3. The high degree and long duration of the immunity gained by prophylactic inoculations.
4. The possibility of immunization against pure parasites.
5. The facility with which the inoculation material is preserved.

The disadvantages, however, may be summarized as follows:

1. The manufacture of the inoculation material is rather complex and with some pathogenic bacteria (pest), not without danger.
2. The increased susceptibility during the interval between the inoculation and the onset of Immunity.

The last point applies not only to aggressins, but equally to other methods of active immunization. In times of an epidemic, aggressin immunization should never be undertaken.

When one bears in mind the great advantages derived from the employment of this form of immunization, its extensive use should be expected; especially so as animal experimental work with the most important of infectious bacteria: typhoid, cholera (Bail), colon (Salus), dysentery (Kikuchi), staphylococcus (Hoke), has proven it to be of greater or less success. And it is therefore no false prophecy, to say that this method will be employed more and more frequently in the future; particularly for pest, in man, results obtained in animal experimentation by Hueppe and Kikuchi have more than sanctioned its employment.

Other methods of immunization based upon the Aggressin principles have been advocated, but none have attained any practical significance.

Brieger's Bacterial Extracts.

Mention however must, in passing, be made of the work of Brieger and his co-workers Mayer and Bassenge. Brieger had made extracts of typhoid and cholera bacilli, in the main identical with artificial aggressins. As far as his sterilization was concerned, he obtained that by filtering the extract through the Pukal filter. One should remember that during this procedure many important substances are lost, but in spite of this, his results of inoculation in man have been most encouraging, and there is a possibility that his method may take the place of Wright's or Pfeiffer and Kolle, as the reactions are very much milder.

Entirely different from the extracts of living bacteria are those made from previously killed ones. Neisser and Shiga among others, have immunized against half parasites in this manner. This is not surprising as the dead bacter al bodies can be similarly used for this purpose. As a general rule, wherever dead bacterial bodies cannot be used for immunization, their extracts will also be found inefficient. The oldest bacterial extracts in use are the tuberculins.

CHAPTER V.

TUBERCULIN DIAGNOSIS.

As a member of the class of bacterial extracts, tuberculin merits especial consideration, because it is used not only for immunization, but also for diagnostic purposes. Tuberculin diagnosis can be employed in three ways.

1. As Koch's subcutaneous method.
2. As the cutaneous reaction (v. Pirquet) and ointment reaction (Moro).
3. As ophthalmo reaction (Calmette).

Koch's Subcutaneous Method.

In the chapter on aggressins it was shown that when a normal animal was inoculated with a certain definite quantity of bacterial extract, it could readily withstand any effects of such inoculation. If, however, a similar quantity was injected into an animal previously infected with the same bacterium, dangerous symptoms would be in evidence and if the dose were large enough, death would be likely to follow.

With these facts for reference, the following experiments will be easily understood. A number of tuberculous guinea-pigs, and a number of normal ones as control, are injected with varying doses of tuberculin. After twenty-four hours some of the tuberculous animals are dead, others very ill, while the normal guinea-pigs remain perfectly active. Just as in the aggressin experiment, we have here a bacterial product in itself possessing only slight toxic qualities which has so increased the virulence of the infection already existing, that an ailment which is usually of a slowly progressive nature becomes transformed into an acute one, terminating in the death of the animal.

The close analogy between the experiments with aggressin as the injected substance, and that of the tuberculin, will become more clear when the nature of the latter is perfectly understood.

Derivation of Tuberculin.

Four to six weeks old pure cultures of the tubercle bacilli grown in 5 per cent. of glycerin bouillon are filtered, and the filtrate then evaporated down to 1/10 of its original volume. The resultant fluid, known as tuberculin, is dark brown and syrupy in nature, and has the quality of keeping indefinitely.

It consists, therefore, as we see of a 50 per cent. glycerin extract of the soluble products of metabolism of the tubercle bacillus.

A part of the glycerin has, however, been used up for the nutrition of the bacteria and thus it is highly probable that after four to six weeks the bouillon contains less than 5 per cent. glycerin and the evaporated solution less than 50 per cent. The specific substances contained within the tuberculin have not been definitely established. As probable elements, however, may be recorded, products of secretion of the living bacteria, of degeneration of the dead bacilli and finally the glycerin soluble substances extracted from the bacterial bodies during the heating. All these substances no doubt, and many others about which we lack information, are directly concerned in the activity of the tuberculin.

Among the many unsolved questions which here present themselves may, in addition be mentioned the one, to the effect: whether any substances exist in the filtrate which are thermolabile, and therefore destroyed or modified by the heating? According to Bail's researches, the aggressin of the tubercle bacillus differs from all other aggressins in that it is not thermolabile and can moreover withstand high grades of temperature. In spite of this though, attempts to eliminate the heating during the manufacturing of the tuberculin should merit consideration.

If merely the term "Tuberculin," is used one always has in mind the filtrate tuberculin, also known as Old Tuberculin.

The above described experiment with the tuberculous guinea-pigs has its analogy in the use of tuberculin in the case of man. Here, however, in order to avoid dangerous symptoms far smaller doses of tuberculin are selected.

The Tuberculin Reaction in Man.

If therefore of two individuals one is tuberculous and the other not, and both are injected with the same amount of old tuberculin 0.001 c.c., the healthy individual remains perfectly normal while the tuberculous person shows a typical symptom complex which can be described under,

1. General reaction.
2. Focal reaction.
3. Local reaction.

The *General Reaction* consists of, *fever, headache, malaise, nausea, insomnia, cough irritation, palpitation*, etc. The most constant symptom is *increased temperature;* the other manifestations may only be very mild or even entirely absent.

The *Focal Reaction* exhibits evidences of a fresh inflammatory process in the suspicious or old tuberculous foci. In cases of lupus, laryngeal, and iris tuberculosis, this inflammatory reaction can be distinctly seen. In pulmonary tuberculosis the previously vague physical signs may now become definite; rales may appear, dulness may be increased, and eventually pains in the chest may arise.

The *Local Reaction* is noticed at the point of inoculation. In spite of the sterile needle and thorough disinfection, the skin around the site of the injection becomes red, swollen and painful. That this is not due to dirt *infection* is proven by its absence in non-tuberculous individuals.

Of the three types of reaction the general and focal symptoms are the most constant. Both are so characteristic for the existence of tuberculosis, that their appearance justifies the diagnosis. In practice, however, it is the *general reaction, or almost exclusively the manifestation of fever*, which is taken as the guiding symptom.

The focal reaction in all non-visible tubercular lesions is determined by subjective methods, while increase in temperature is alone an objective finding.

In carrying out the tuberculin test, one must remember several practical points which are of help for the correct interpretation of the results. These may be summed up thus:

Inasmuch as the rise of temperature is of diagnostic importance, *no patient with any fever should be subjected to the inoculation.* For several days previous the patient's temperature should be taken every three hours and only if the temperature does not exceed 37° C. per axilla should the tuberculin diagnosis be undertaken.

The *quantity of tuberculin to be injected is also of the utmost consequence.*

Dosage of Tuberculin.

Too high doses should be avoided, as the specificity of this reaction, like all other biological reactions, is limited quantitatively. While small doses of tuberculin will give a rise of temperature only in tuberculous individuals, larger doses may give the same rise even in healthy people. In addition, too large doses as a rule may produce a general reaction which might be very severe and entirely injurious.

The dosage advised by Robert Koch for the diagnostic tuberculin reaction is as follows:

1. 0.0001 c.cm. T. (for very weak individuals and children).
2. 0.001 c.cm. T.
3. 0.005 c.cm. T.
4. 0.01 c.cm. T.
5. 0.01 c.cm. T.

The dose chosen at the first injection is as a rule 1 mg. T. Very weak individuals, *i.e.*, those in an advanced stage of tuberculosis or those who have experienced a recent hemoptysis, as well as children should receive an initial dose of only 0.1 mg. T. Bandelier and Röpke who have a wide experience in this field, advise 0.2 mg. T. as the primary dose.

Few patients show a distinctly positive fever reaction even with this small dose; *by a positive reaction is meant an increase in the temperature so that the latter is at least* 0.5° *C. higher than the highest point before the injection.* If the temperature has not increased, the reaction is negative, and after an interval of two to three days of normal temperature the second inoculation of 5 mg. T. is given. If it happens occasionally that after first inoculation there is a doubtful reaction, *i.e.*, there is an increase of 0.2° to

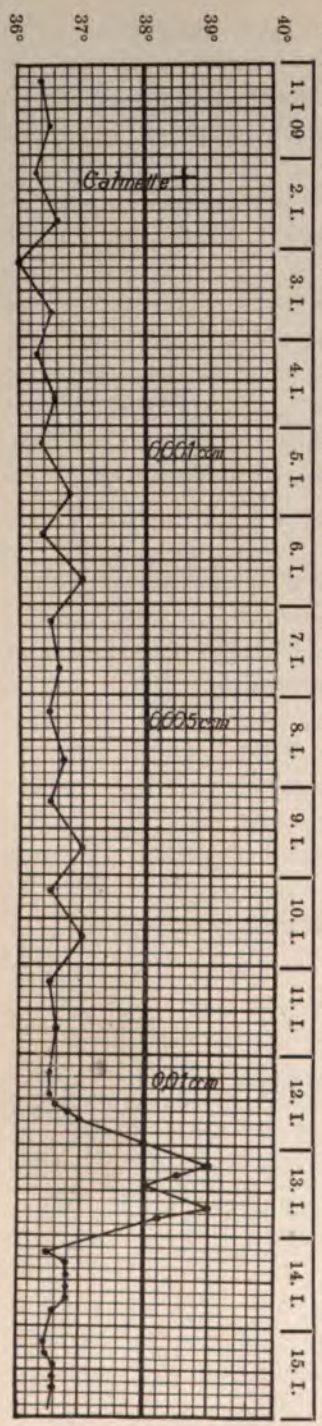

CHART 1.—Example of a diagnostic tuberculin reaction.

0.3° C. then the dosage at the second injection should not be increased to 5 mg., but the same amount 1 mg. T. is to be repeated. In a tuberculous individual this repeated injection of 1 mg. frequently results in a distinctly positive reaction, while in a non-tuberculous patient instead of the former doubtful, a distinct negative reaction is obtained.

The general rules given for the first inoculation also apply to the second with 5 mg. In a doubtful reaction with this dose, one does not directly proceed to the 10 mg. dosage, but the 5 mg. dose is repeated and only after a negative reaction with the repeated 5 mg. dose are the 10 mg. injected (see accompanying Chart 1). This represents the maximum amount of tuberculin to be used for diagnostic purposes. Koch advises repetit on of this dose if no reaction is obtained. The majority of authorities, however, abstain therefrom. In fact some investigators claim that a reaction obtained after inoculat on of 10 mg. cannot be considered specific, because there is a class of non-tubercular individuals that responds to this quantity of tuberculin.

Most tuberculous persons react after a dose of 1 to 5 mg. T.; those, however, who are very far advanced or who suffer from severe cachexia, remain unresponsive to even much greater doses; in addition, patients whose serum contains antituberculin, do not react because the inoculated tuberculin is quickly neutralized.

Loewenstein's Dosage Scheme.

According to Loewenstein the tuberculin reaction does not depend so much upon the quantity of the tuberculin, as upon the frequency that it is injected. He, therefore, advises that the same amount, about 0.2 mg. be inoculated four times during the course of twelve to sixteen days. And the results are that in by far the greater majority of tuberculous patients a typical reaction appears after the third or fourth injection. The author has no personal experience with this method, but the reports of other authorities do not exhibit as favorable results as those claimed by Loewenstein.

Technique of Tuberculin Injection.

As for the technique of injection, the inoculation is always given subcutaneously, and the back or breast is the best site for it. The dilution is made with physiological salt solution or 0.5 per cent. carbolic solution, and it is advisable to make it immediately before the injection.

Value of Reaction.

In interpreting the result of the reaction one must exclude rises of temperature due to extraneous influences such as Angina, Influenza, etc. Furthermore there are individuals, especially hysterical ones, in whom any injection as such is apt to produce a rise of temperature. To guard against such a possibility an injection of physiological salt solution should be made and thus quiet any suspicion of error.

Indications.

The diagnostic use of tuberculin is indicated when one is dealing with adults who present *clinical symptoms, or clinically suspicious symptoms of tuberculosis*, but are devoid of the presence of tubercle bacilli and temperature.

Contra-indications.

Tuberculin is contra indicated in patients with high fever, and during or shortly after hemoptosis or hematuria. In epilepsy, marked cardiac or renal affection, arteriosclerosis, diabetes, and similar conditions, inoculation should be undertaken only under the strictest indications and with great care.

A positive general reaction means that the individual is infected with tuberculosis, but does not throw any light upon the site, the extent, or the prognosis of the infection. The focal reaction allows the diagnosis of the position of the lesion.

The Cutaneous Reaction.

The cutaneous reaction was first introduced by v. Pirquet, who noticed that by scarification of the skin and application of tuberculin, tuberculous children would develop a distinct papule at this point, while in non-tuberculous conditions such a reaction would be absent.

The Technique of the Cutaneous Reaction.

"The patient's forearm on the inner side is cleansed with ether; two drops of the pure undiluted old tuberculin are placed upon the skin about 10 cm. apart, and then the skin is scarified first between the two drops, for the purposes of a control, and next within each of these drops.—[A boring scarifier devised for this, works very easily.] Finally a piece of cotton is placed upon each of these drops and allowed to remain there for ten minutes, after which the cotton is removed. A dressing is not necessary."

Interpretations of the Reaction.

Scarification of itself produces the so-called "*traumatic reaction*," *i.e.*, a small wheel with a rose colored margin appears around each of the three

points of scarification. This reaction passes away after several hours and only a small scab remains surrounded by a red rim.

This "*traumatic reaction*" is to be sharply differentiated from the "*specific reaction.*" The latter is noticed only upon the upper and lower points where the tuberculin has been applied and consists of a red, indurated papule which rapidly extends in size and elevation, measuring 10 to 30 mm. in diameter. (Fig. 1, Plate I). The papule may be round or have irregular margins. Scrofulous children show small, irregularly raised follicular infiltrations around the specific reaction. This is known as the "*scrofulous reaction.*" It may appear as early as within three hours, but usually occurs within twenty-four hours. It arrives at its maximum within forty-eight hours; occasionally it is delayed and may fully develop until the third or fourth day and then it begins to fade. Frequently a small pigmented spot remains. General and focal reactions are practically absent.

Moro's Ointment Reaction.

Moro and Doganoff found that a 50 per cent. ointment of tuberculin in lanolin rubbed into the skin without scarification, would give a reaction which consisted of small nodular or papular efflorescences after the nature of Lichen Scrophulosorum. Therefore, in accordance with the number and size of these nodules as well as the time of their appearance, three grades of reaction are described.

FIG. 13.—Inoculation with tuberculin for the Pirquet reaction.

In carrying out the reaction the ointment is heated to 25° C. and a quantity about the size of a pea is thoroughly rubbed into the skin of the abdomen or the region of the mamilla, for almost a minute. The diagnostic value of the reaction is variously interpreted.

An almost analogous reaction, described independently of Moro, by Lignières and Berger is to be found in thoroughly rubbing in concentrated old tuberculin into the shaved skin of tuberculous cattle.

The Ophthalmo Reaction.

Historical. At the discussion which followed v. Pirquet's presentation of his cutaneous reaction, Wolff-Eisner remarked, "that by instilling some 10 *per* cent. tuberculin into the conjunctival sac, a local conjunctivitis was

obtained and occasionally, also a general reaction. The marked severity of the reaction, however, and its apparent lack of specificity, made its diagnostic value improbable." Calmette, who believed that Wolff-Eisner's failure in obtaining authentic results lay in the fact that glycerin was contained in the old tuberculin employed by him, went to work and by alcohol precipitation obtained a glycerin-free dry product, which he used in a 1 per cent. solution equivalent to 10 per cent. old tuberculin. It was he, therefore, who first established the clinical diagnostic value of the reaction. But his hypothesis was erroneous, as the mild reactions which he obtained were not due to the absence of glycerin, but because the Lille tuberculin made use of is much weaker than the German preparation. The author was able to show that the old tuberculin could very well be used for the Ophthalmo reaction if instead of the 10, a 1 per cent. dilution was made. Thus employed, the reaction is exceedingly mild and specific. Eppenstein later advised a 2 and 4 per cent. dilution in cases where the 1 per cent. solution gave no reaction.

Technique of Reaction.

It is of extreme importance to have freshly prepared sterile dilutions of the old tuberculin (Höchst Farbwerke). *All the ready-for-use preparations on the market should be discarded. This applies also to the "Tuberculin Test" Calmette's* sold by *Poulenc Freres.*

The mishaps and low grade of specificity often ascribed in literature to the ophthalmo reaction can in a greater majority of cases be explained by the employment of preparations other than the 1 to 2 per cent. fresh dilutions of the old tuberculin advocated by the author, and in still another number of cases to its employment in conditions where it was distinctly contraindicated. The preparation of fresh tuberculin dilutions is very much simplified by the "*Ophthalmodiagnosticum for Tuberculosis,*" of the firm P. Altmann, Berlin N. W. 6 (Fig. 14).

FIG. 14.—Ophthalmodiagnosticum for tuberculosis. (*After Citron.*)

This outfit consists of twelve sealed glass tubes each containing 0.1 c.c. old tuberculin; a cylinder for the dilution graduated in percentages, and a measuring pipette 0.1 c.c. in size fitted with a rubber bulb. One of the sealed ampoules is shaken so that the tuberculin is collected into its broader part and then broken at the designated point near the narrow end. The tuberculin is drawn up to the mark into the pipette and then transferred into the cylinder. Boiled water or sterile saline is added to the 1, 2 or 4 per cent. dilution mark. The pipette is washed clean in the solution by successive aspiration and expulsion in order to free it completely of the remaining concentrated tuberculin, and can now be employed as the eye dropper.

The solution should be used only on the day it is prepared. The tuber-

culin in the sealed tube can be kept indefinitely. The pipette and graduate are sterilized by dry heat, boiling or by thorough washing in boiling water.

One drop of the tuberculin dilution is deposited into the inner angle of the eye, and care should be taken that the drop is not immediately expelled, but evenly distributed in the conjunctival sac.

Gradation of the Ophthalmo Reaction.

In tuberculous individuals the reaction appears in twelve to twenty-four hours, and according to its intensity can be divided into three grades.

First Grade.—Reddening of the caruncle and inner side of the lower lid (+) (see Fig. 2, Plate I).

Second Grade.—Same as above but additional involvement of the conjunctiva of the eyeball (+ +).

Third Grade.—Conjunctivitis purulenta, phlyctenulæ and other such severe manifestations (+ + +).

The reactions of the first and second degree occur most frequently. The manifestations associated with the former of these are so mild that the patient himself does not usually notice them. If the proper dilution is used and the contraindications of this test are observed, a reaction of the third degree is obtained only in exceptional cases. Fever never occurs. The other eye serves as a control. It is advisable therefore before undertaking the reaction, to note carefully any differences that may exist in the conjunctivæ on both sides.

Selection of Correct Dilution.

It must be remembered that 1. The greater the dilution, the more specific is the reaction.

2. The test should not be repeated upon the same eye, even if there was no reaction at all at the first instillation.

The following procedure should be adopted. A drop of the 2 per cent. tuberculin dilution is placed within the left eye. If a positive reaction takes place, it is of great probability that the patient is suffering from an active tuberculous process and thus the diagnosis is established. If, however, that proves insufficient, and further corroboration is required, the patient should receive after the first reaction has entirely subsided one drop of a 1 per cent. tuberculin dilution into the right eye.

If a negative reaction is obtained at the instillation of the 2 per cent. dilution, one drop of the 4 per cent. dilution is placed into the right eye. A negative reaction with the 4 per cent. mixture speaks almost conclusively for the absence of tuberculosis except in far advanced cachectic conditions. A positive result does not, on the other hand, indicate the presence of tuberculosis, as there are many normal individuals who react to a 4 per cent. tuberculin concentration.

Indications for Ophthalmo Reactions.

The ophthalmo reaction is indicated in all suspicious cases of tuberculosis where the presence of bacilli cannot be demonstrated and where the subcutaneous reaction either on account of the presence of temperature or other reasons cannot be undertaken.

This test is much milder and more agreeable to the patient than the subcutaneous one, and in ambulatory work more significant, inasmuch as it does away with any necessity for considering as a guide the temperature taken by the untrained and usually unreliable patient.

Contraindications for the Ophthalmo Reaction.

The ophthalmo reaction is contraindicated in all diseases of the eye, tuberculous or otherwise. If one eye only is affected, the reaction should not be undertaken upon the healthy eye. Similarly, patients who have had some eye disease, even though many years ago, those who by reason of their occupation are readily exposed to eye diseases, or who live in districts where trachoma is prevalent should be excluded from the test. The reason being that in those individuals the conjunctival mucous membrane becomes a locus minoris resistentiæ and therefore easily inflamed

Repeated instillations of tuberculin into the same eye, may set up very severe disturbances. Scrofulous children often show reactions of the third degree, inasmuch as they possess the constitutional tendency which makes them easily susceptible to conjunctivitis or phlyctenulæ. In patients with a positive ophthalmo reaction which has subsided, a recurrence of the conjunctival inflammation is frequently observed when they begin to receive subcutaneous inoculations of tuberculin for therapeutic or even diagnostic purposes.

The Specificity of the Tuberculin Reaction.

The one real essential for the practical application of all biological reactions, is the specificity of the same. There is, however, as will be repeatedly pointed out further on, no single absolutely specific reaction. In fact, it would be more exact to consider these biological reactions only relatively specific; the latter depending upon the quantity of the required antigen and the reacting organism. In this connection it may also be said, that it is never possible to draw an exact line between the specific and non-specific biological reactions. There always will be a doubtful zone. As a general rule, however, it may be said that the smaller the quantity of antigen that is required and the stronger the resulting reaction, the more probable is the biological specificity.

In tuberculosis this problem is rendered still more complex by the pathological anatomical findings, whereby it is shown that an extraordinary high percentage of individuals have undergone tubercular infection at some time during life. The clinical consideration of tuberculosis, however, does not deal with the diagnosis of these harmless, practically healed tuberculous foci; what the clinician desires to know is whether or not a group of symptoms manifested in a patient is of a tuberculous nature or not. In other words, it is not the latent, inactive, but the active form of tuberculosis that is to be diagnosed. If, therefore, one views the various tuberculin tests from such a stand-point as this, he arrives at very material differences.

The reaction of least specificity in adults is the v. Pirquet's cutaneous reaction. In children it is far more specific.

Apropos this latter, v. Pirquet makes some very interesting observations.

Out of 747 children in Escherich's clinic in Vienna upon whom the reaction was tried, there were:

clinically tuberculous	130,	out of which	113 (87%)	showed a positive reaction;
clinically non-tuberculous	512,	" " "	104 (20%)	" " " "
doubtful	115,	" " "	56 (48.6%)	" " " "

Almost all of the tuberculous children who did not react were cachectic.

As for the positive reaction in non-tuberculous cases, the age of the child in large part explains the great differences found.

Whereas healthy infants up to the sixth month almost never give a positive reaction, healthy children of

1 to 2 years react in 2 per cent. of cases.
2 to 4 years react in 13 per cent. of cases.
4 to 6 years react in 17 per cent. of cases.
6 to 10 years react in 35 per cent. of cases.
10 to 14 years react in 55 per cent. of cases.

In adults one meets with a positive v. Pirquet's reaction in more than 70 per cent. of all cases. V. Pirquet explains this by the presence of latent tuberculosis.

It therefore becomes self evident, that the cutaneous reaction in adults is void of any diagnostic value. A negative reaction only, can be fully relied on, and that, if no cachexia exists.

In young children on the other hand, v. Pirquet's method should be the choice. In addition to its being entirely harmless, and easily applied, it possesses a high diagnostic value.

As for *Koch's subcutaneous reaction*, it is specific, inasmuch as it is a rare exception to get a negative reaction in an active tuberculous process. This occurs only in cases either with very severe cachexia or those with freely circulating anti-tuberculin in the blood. If the latter two possibilities are excluded, the absence of a positive reaction speaks decidedly in favor of the absence of tuberculosis.

The interpretation of a positive reaction as to the existence of clinically active tuberculosis cannot be so definitely answered. From the work of most of recent authorities, however, it seems to be taken for granted that a positive reaction does mean an active tuberculosis; still, this statement requires a great deal of consideration and limitation as well.

In this connection the statistics of Franz are of great interest. Out of 400 apparently healthy soldiers in one of the Austrian regiments who in 1901 —their first year of service, received an inoculation of 0.003 c.c. of tuberculin, a positive result was found in 61 per cent. of the cases. In the following year (1902) 100 of the soldiers were re-inoculated and all of those who reacted positively the first time, did so a second time, in some instances even though the second dosage was smaller. Moreover, fourteen others who

responded negatively the previous year showed positive results this time, making a total of 76 per cent. Out of 323 men inoculated for the first time in 1902, 68 per cent. reacted positively. It must be mentioned, however, that the majority of members of this regiment came from a very tuberculous district. The same author also examined a Hungarian regiment in a tuberculous-free district, and under similar circumstances found a positive reaction in 38 per cent. of cases. Although these figures may be exceptionally high, they are without doubt conclusive as to the fact that Koch's reaction in this respect, cannot be considered specific for "active" tuberculosis. Franz in addition gives important statistics concerning the health of the inoculated soldiers whom he examined for years following the inoculation. The following charts taken from the most recent publication of Franz (Wien. Klin. Woch., 1909, No. 28) tabulates what has been said above.

Regiment.	Year of injection	No. of soldiers inoculated.	Positive reaction. / Negative reaction.	During the period of three years, those that terminated their service through death, invalidity or longer leave of absence showed. Tuberculosis.	Disease suspicious of tub.	Other diseases.
Bosn. Inf. Reg. No. 1.	1901	400	+ 245 (61%)	17 (8 deaths)	22	10
			− 155 (39%)	5 (4 deaths)	25	7 (1)
Bosn. Inf. Reg. No. 1.	1902	323	+ 222 (68.7%)	13 (6 deaths)	28 (1)	7 (2)
			− 101 (31.3%)	4	13	5
Inf. Reg. No. 60.	1902	279	+ 108 (38.7)	4	4	8
			− 171 (61.3)	3 (2 deaths)	5	12
Total.....		1002	+ 575	34 (14 deaths)	54 (1)	25 (2)
			− 427	12 (6 deaths)	43	24 (1)

Regiment.	Time of observation.	No. ill with manifest tuberculosis.	Those who reacted in 1901 and 1902 to 3 mg. tuberculin. Positive.	Negative.
Bosn. Inf. Reg. No. 1. I Ser. (400 men).	From 10. x. '04 until end of 1908.	10	6	4
Same. II Ser. (323 men); Inf. Reg. No. 60 (279 men).	From Oct., 1908, until end of 1908.	6	5	1
		2	1	1
		18	12	6

Specificity of Ophthalmo Reaction.

In regard to the specificity of the ophthalmo reaction, the conditions here are more favorable than in both of the preceding tuberculin tests. The following short chart is explanatory. Positive reactions were obtained in

	Audéoud.	Petit.	Citron (1st series).	Eppenstein.	Schenk and Seiffert.
Tuberculosis.....	94.6%	94.3%	80.7%	72.3%	78.6%
Suspicious cases..	81.0%	61.6%	80.0%	40.0%	30.0%
Normal cases....	8.3%	18.4%	2.2%	9.0%	5.8%
	Calmette's preparation.		1% old tuberculin.		

It is evident from the above figures *that by the use of the* 1 *per cent. tuberculin a grade of specificity is reached which can be considered quite high, as the non-tuberculous react only in a very small percentage of cases, while existing tuberculosis is detected in* 80 *per cent. of the subjects.* Clinical examinations of the positive reacting patients show that the latter belong to the group of active tuberculosis. Absolute reliance, however, in the determination as to whether the positive reaction given is due to an active or latent tuberculosis, cannot even be placed upon the ophthalmo reaction.

According to several authors, it is claimed that typhoid fever, rheumatism, and syphilis (in the stage of eruption) are very prone to give a positive ophthalmo reaction, without the presence of a simultaneously existing tuberculosis.

In conclusion, therefore, the author finds it difficult to make any general statement as to the preference of one or the other reaction test for diagnostic purposes.

In children, however, it may be said that the application of the Pirquet reaction, in adults, the ophthalmo reaction, are given preference to Koch's reaction, provided no contraindications exist against the former, and that treatment with tuberculin is not to be undertaken. In the latter instance, the recurrent ophthalmo reaction when the tuberculin therapy is instituted, authorizes the use of Koch's subcutaneous diagnostic method.

Mallein, Trichophytin.

Similar to old tuberculin, the Mallein (Helmann and Kelning) has been obtained from cultures of Glanders bacilli and the Trichophytin (Plato) has been isolated from the Trichophyton fungi. Mallein has already attained a place in practical application for the diagnosis of glanders in veterinary medicine. Like tuberculin it is harmless in normal organisms, but brings about temperature and a local reaction at the site of the injection when inoculated into glanders stricken animals. Various general symptoms may also appear. Its employment in a manner analogous to the ophthalmo reaction *is also possible.*

CHAPTER VI.

The Tuberculin Therapy.

Right at the beginning it must be made clear, that the use of tuberculin is not to be considered as a curative agent against tuberculosis, but rather in the light of a *bacterial extract for active immunization.* In the previous chapter it has been shown that while there are some infectious diseases where immunization can be accomplished by the use of bacterial extracts and dead bacteria, there are others where immunization is possible only when living vaccines or aggressins of living bacteria are employed. In both of these instances, however, healthy individuals are being treated to be protected from future infection. An exception is presented by rabies. In this disease, the vaccination against the active symptoms is instituted after the infection has already taken place, but the redeeming feature about its treatment is the existence of the very long incubation period. Therapeutic use of tuberculin, however, is a form of active immunization which belongs to neither of the above classes. The principle involved here is entirely different, and the question arises if it is at all possible to obtain an active immunity by the injection of antigen in a condition where infection has already taken place, and produced pathological changes. [In other words, where spontaneous immunization has failed.]

An answer to this question is to be found in Koch's fundamental experiments which have been the basis as well as starting point of the entire tuberculin study.

If a normal guinea-pig is inoculated with tubercle bacilli, the point of inoculation very soon closes. After ten to fourteen days there appears at this site a small hard nodule which finally ulcerates. This shows no tendency to heal and remains so until the death of the animal. If, however, an already tuberculous guinea-pig is similarly inoculated, while the point of inoculation also closes, no indurated nodule appears. Instead, a necrotic process of the skin sets in after the second day, which finally terminates in the casting off of the slough and the formation of a flat ulceration that heals rapidly. It does not matter at all whether living or dead tubercle bacilli are used for the second infection.

In explanation of the above phenomenon it must be said that the first injection although it had a fatal effect upon the animal must have stimulated certain immune reactions within the organism which became manifest after the second inoculation. That a condition similar to this, or even more favorable exists in man, is proven by the fact that while the large majority

of people become infected with tuberculosis at some time during their lives, only a small proportion show symptoms referable to the disease and the other greater number undergo spontaneous cure.

Koch further showed that the injection of tuberculous guinea-pigs with large doses of tubercle bacilli produced rapid death, while frequently repeated small doses, evinced favorable effects upon the site of injection and the general condition of the animals. In this way he proved the beneficial influence which successive inoculations exert upon the primary infection.

In the employment, however, of dead tubercle bacilli in man for the purpose of therapeutic injections, a serious difficulty presented itself. It was found that the inoculated dead bacilli were not absorbed, but remained for a long time at the seat of the inoculation instigating suppurative processes. On intravenous application, formation of tubercular nodules was noticed.

Koch realized that these harmful effects were due to the non-absorbable parts of the tuberc'e bacilli; in the main the bacterial capsules. He therefore attempted to extract the immunizing substances, and in this way brought about the existence of old tuberculin.

Questions may here be asked to the effect, whether this old tuberculin is identical with tuberculous antigen; whether it is at all a feasible preparation for purposes of immunity; does it contain all the important elements of the tubercle bacillus, if not which are lacking? The specificity of immunity reactions has already been dwelt upon sufficiently to make it clear that immunizing a healthy individual with old tuberculin will bring about an immunity only against the substances contained within this preparation. That that does not meet the requirement is proven by the fact that an animal immunized against tuberculin will not be protected against a later infection with living tubercle bacilli. It cannot therefore, be expected that immunization of a tuberculous individual with old tuberculin will protect him against living tubercle bacilli. The expectation, however, that his immunity will be raised against old tuberculin only, is fully borne out.

Furthermore, we have seen that in the aggressin experiments, inoculation of animals with the aggressin antigen was sufficient to increase the immunity so that a subsequent infection was not attended by any harmful effects. In this case the injected living bacteria are not destroyed, but their ill effects upon the immunized organism have been paralyzed. In other words, the parasites have been transformed to saprophytes. That a similar state of affairs exists in the use of antitoxic sera will readily be seen. The antitoxic diphtheria serum, for example, neutralizes the toxin and thus cures the disease. The bacteria themselves, however, remain intact and also infectious for untreated individuals. Only later on are they absorbed by the phagocytes. When therefore in an individual who has passed through a course of tuberculin treatment there are found fully virulent tubercle bacilli in the sputum, it is no proof, if that is the only corroborative evidence, that the tuberculin treatment had been inefficient. In fact, there are strong possibilities that the tubercle bacilli have become transformed into saprophytic bacteria. It is, however, a noteworthy and important fact, that immunization with tuberculin proves no protection against later infection with living tubercle bacilli, while in the case of aggressins and toxins this is possible.

Although tuberculin cannot be considered as the aggressin or toxin of the tubercle bacilli, it simulates these substances with sufficient closeness to

warrant its use in tuberculosis. It brings about an immunity against some of the poisonous products of the tubercle bacillus, leaving the others to be combatted by the natural fighting powers of the individual.

Various Tuberculin Preparations.

The knowledge that this old tuberculin represents only a partial aggressin, or toxin, and by that is meant that it does not contain all the necessary elements for the establishment of a true immunity, has led to the production of a large group of preparations which are supposed to supply the missing properties of the old tuberculin.

The most important of these preparations was made by Robert Koch. Those which are of frequent use are:

a. Old tuberculin (T. Tuberculin)—preparation described on page 47.

b. Original old tuberculin (T. O. A. Tuberculin Original Alt.)

The latter consists of the original filtrate of the tubercle bouillon culture and varies from the old tuberculin in that it is not heated and reduced to 1/10 its volume. The omission of heating is certainly not without effect, inasmuch as high heat without doubt modifies in some way the soluble bacterial substances. This preparation has not been used therapeutically by Koch himself. Spengler and especially Denys, who have made wide use of it under the name of "Le bouillon filtré," have been its main supporters.

c. Vacuum tuberculin (V. T.) is the original tuberculin which has been reduced in vacuum to 1/10 its volume.

d. The aqueous tuberculin Maraglianos (Tuberculina Aquosa) is closely allied to the above tuberculins. It contains all the water soluble extracts of the living tubercle bacilli obtained by extraction of the living bacteria in distilled water, followed by filtration. As is evident, it is prepared on the same principle as Brieger's bacterial extracts and Wassermann-Citron's artificial aggressins.

The above mentioned tuberculin preparations are all very much alike in that they contain the soluble bacterial elements. Their action therefore corresponds more or less to that of old tuberculin.

New Tuberculin Preparations.

Another set of preparations have as their basis the insoluble bacterial substance which cannot as such, in either living or dead form, be absorbed. Since, however, the absorption of bacteria is a prerequisite to their proper action, it was necessary to so alter the body substances of these bacteria that they could be taken up. Koch found that this was best accomplished by thoroughly pulverizing the bacilli in large mortars. And by this means the first preparation which he obtained was

e. New tuberculin T. R. (Koch) Tuberculin Rückstand or Residual Tuberculin.

The technique is carried out by making cultures of young tubercle bacilli which are then thoroughly dried in vacuum and finely ground in mortars. The pulverized bacilli are agitated in distilled water and the turbid mass is centrifugalized. The sediment thus obtained composes the T. R. or the tubercle bacilli residue.

T. R. therefore contains the aqueous insoluble components of the tubercle bacillus, while the soluble ones are retained in the opalescent supernatant fluid which Koch calls TO (Tuberculin Original).

T. R. is readily assimilated by patients. If carefully administered it produces very little infiltration and only slight temperature and general reaction. Its price is comparatively high (1 c.c. costs 8.50 marks).

The first preparation which contained both the soluble and insoluble elements of the living bacilli was the

f. New Tuberculin—Bacilli emulsion (B. E.) which consists of T. R.+ T. O.

The living tubercle bacilli are first pulverized in a mortar and then suspended in salt solution. No centrifugalization is necessary, but sedimentation is adhered to, and besides, 50 per cent. glycerin is added for preservation purposes. Next to T. the new tuberculin B. E. has been most carefully studied.

Equally lacking in being an ideal antigen is the B. E. inasmuch as immunity attained by its injections is not at all proof against subsequent infection.

Closely resembling the B. E. is

g. the Tuberculin Béraneck.

Béraneck produced two tuberculin preparations of which one is in the main identical with TOA, while the other is an extract of tubercle bacilli with 1 per cent of phosphoric acid. Both of these tuberculins are mixed together and applied. Sahli reports good results with this mixture.

Action of Tuberculin.

Although none of the described tuberculin preparations can be considered a true antigen for the tubercle bacillus, they have nevertheless an undoubtedly favorable effect upon tuberculous individuals. To a certain extent the benefits must be said to be derived by the mechanism of partial immunization. This in itself does not, however, explain the entire phenomenon of their successful action.

On examination of the tuberculous organs of animals treated with tuberculin, there will be found within the healthy tissue surrounding the tuberculous foci, a fresh inflammatory reaction. This consists of a serofibrinous exudate and a zone of leucocytes intruding to a certain extent upon the tubercular lesion. *Tuberculin acts only upon tuberculous tissue which is still alive and not upon dead, cheesy or necrotic structures.*

If enough tuberculin is given so that death of a tuberculous guinea-pig occurs, the changes found are striking. On dissection, about the point of inoculation Koch reports a marked congestion of the blood vessels giving a red and often an almost dark violet appearance. This discoloration extends for a greater or less distance from the site in question. The neighboring lymph glands are similarly reddened. Besides the tuberculous changes present within the liver and spleen, these organs show on their surface many blackish-red spots varying in size from that of a pin-point to a hemp seed, and resembling very closely the ecchymosis found in some infectious diseases. On microscopical examination are found no blood extravasations, but very widely distended capillaries directly surrounding the tuberculous foci. The capillaries are so densely plugged with red blood cells that it seems almost impossible for the circulation to have continued in these places. In exceptional cases only, are the blood vessels ruptured and the escaped blood found within the tuberculous foci. The lung presents similar changes,

but not as regularly or of such characteristic appearance. The small intestine is often deeply and evenly congested. In all this symptom-complex, in short, the never failing and almost pathognomonic feature is the hemorrhagic-like spots on the liver surface.

Koch considered that the tuberculin brought about the death of the tuberculous tissue. He furthermore interpreted the disappearance of the reaction after inoculations with tuberculin, as evidence that the entire tuberculous structure had been destroyed; in other words that healing had set in.

Accordingly, in the first tuberculin era, the erroneous tendency arose to consider those tuberculous patients as cured who after gradually diminishing reactions to tuberculin had become entirely refractory to it. Truth to say, these individuals had merely become immunized against old tuberculin, and had another preparation such as new tuberculin been injected, a reaction would have recurred.

Basing their conclusions on experimental work, Wassermann, Bruck and also the author have shown that besides the factor of partial immunization, it is the *focal action* of the tuberculin which is the beneficial agent in its therapy.

The inflammatory hyperemia produced, leads to a destruction of the tuberculous tissue, while at the same time the inflammatory process recedes. In addition there is a formation of connective tissue which encapsules the focus and with it also, is associated the local stimulation of antibodies.

The Technique of Tuberculin-therapy.

Three distinct periods can be noted in the history of this therapy. The first began in the memorable year, 1890, when Robert Koch made known his discovery of tuberculin. At this time, the aim of tuberculin treatment was to cause very marked reactions and to continue with the injections until no further reaction was obtained. In lupus, glandular or bone tuberculosis 10 mg. was the initial dose. In tuberculosis of the lungs 1 mg. was the beginning. If the patient reacted to this amount, he received *daily* inoculations of this dose until no reaction appeared. Then 2 mg. T. were given and the same procedure repeated. Quite frequently, depending upon the strength of the individual concerned, 10 mg. was given as the primary inoculation in phthisis, and then rapidly increased. While Koch himself very soon recognized that this rather severe treatment was suitable only for incipient or moderately advanced cases, very sick and far advanced phthisis patients were similarly treated by many physicians. Following such procedure, decidedly unfavorable results were obtained in the latter class of patients and consequently a marked waning in the enthusiasm which first greeted the tuberculin therapy was the inevitable outcome. Thus the once highly praised remedy was entirely rejected.

During the second period only very few former followers of Koch continued their studies in this field. These, however, made it their business to investigate the causes which led to the failure of tuberculin therapy. Their researches led to new principles in the treatment, and to more exact knowledge of its indications as well as contraindications.

The success obtained by the untiring efforts of these investigators brought about after many years a revival of the interest in this therapy. It was again taken up (third tuberculin era) and there is no doubt that when properly handled, tuberculin in well

selected cases is of decided benefit. Nevertheless, even at the present day, conclusive opinion as to many of its details cannot be formed. Attempts are being made to set the individual treatment upon a biological instead of upon the more or less schematic basis thus far employed.

Old Tuberculin (*Tuberculin Koch T.*)

While it was the aim in the early era of tuberculin treatment to produce very strong general reactions, it is the general concensus of opinion at present that it is best to so arrange the tuberculin therapy as to avoid a general reaction and especially the fever. Gœtsch was the man who first called attention to this.

With such object in view, one must begin with small doses. Some men start with 1/100 mg., others with 1/10 mg. T. If no reaction is incited, the dose is increased in five to seven days to 5/100 mg. and then to 1/10, 2/10, 4/10, 8/10, 1, 2, 4, 6, 8, 10, 20, 40, 80, 100, 150, 200, 300, 400, 600, 800, and 1000 mg. This last amount represents the maximum dose. If the patient still gives a focal reaction with such a dose, it is best to repeat it at intervals of

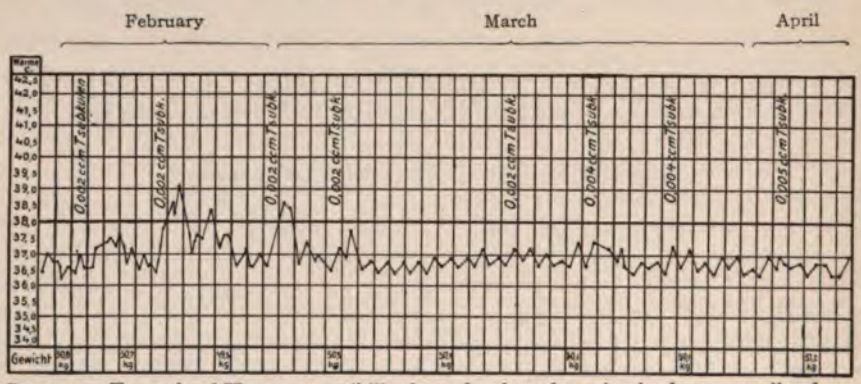

CHART 2.—Example of Hypersusceptibility brought about by going back to a smaller dose.

two to four weeks, until finally no reaction is apparent. Occasionally one will advance with the doses at a more rapid rate, but in general, inoculations should not be repeated more than twice a week.

If at any time a distinct or even doubtful reaction occurs, it is absolutely necessary to await the complete subsidence of the latter, and then the same dose is to be repeated. In such a case, an interval of at least eight to ten days should elapse. The dosage should under no condition be diminished, as thereby instead of immunity, hyper-susceptibility is the result. Therefore, patients who at a short time previously had a diagnostic tuberculin test performed upon them, should receive the highest dose employed in this connection as the initial prescription for their tuberculin therapy. The following chart illustrates the condition of hyper-susceptibility occasioned by a diminution in dosage. (Chart 2.)

Patient had a localized one-sided apex tuberculosis. At a diagnostic tuberculin injection, he reacted only when 0.01 c. cm. T. was employed. After the interval of a month the patient was advised tuberculin treatment. Contrary to the rule just cited, he received as a first injection not 0.01 tuberculin but 0.002 c.cm. T. With this small dose he already had an increase of temperature, although coming rather late, and not quite typical. After this reaction had disappeared, without any other manifestations, the same dose of 0.002 c. cm. tuberculin was repeated and as evident from the chart, a very marked response was inaugurated. This was accompanied by a chill, vomiting, headache, general pains and weakness. In addition there was a slight relapse after the aforementioned symptoms had disappeared. In order to immunize this patient against his hyper-susceptibility, it was advisable to repeat the dose of 0.002 c.cm. T. at which the reaction reappeared, but in a very much milder form. It was only after the fifth inoculation of the same dose that no reaction was in evidence. Thus was the hyper-susceptibility overcome and the patient treated in the general way.

The danger of hyper-sensitiveness also exists if the same reactionless dose is too frequently repeated; especially so if the quantities injected are small. *The higher the dosage, the less liable is the occurrence of hyper-susceptibility.*

This question is above all to be considered when after a certain interval, a second course in tuberculin therapy is advised. In general it can be carried out after a period of three months, even though sometimes certain difficulties may be met with. Petruschky strongly recommended this treatment, in successive stages. (Etappenbehandlung). The author is of the opinion that it is best to retain the patient as long as possible at his acquired immunity (tuberculin) by stretching the course of treatment over a long period of time. He therefore repeats an inoculation of the maximum dose, every three or four weeks and when hyper-susceptibility arises, he changes the preparation and begins with a small dose again.

As for the technical details of the treatment, several practical suggestions may be made.

1. The inoculation should, if possible, be given in the morning hours, for a restless night usually follows an injection in the evening.

2. It is best to so arrange the dilutions that the patient receives a fraction of 1 c.cm. at each injection.

3. The site of injection should be alternated between the back and the breast.

4. The temperature should be taken every two or three hours and a chart of the same kept.

5. Disturbances in the general condition of the patient without the presence of fever are to be considered in the light of general reactions just as fever without other disturbances.

6. The patient's weight should be taken regularly every week, and then the dose should be increased provided no loss in weight has taken place.

7. In cases where the pulse increases in rate or becomes poorer in quality,

the treatment should be undertaken very carefully and the pulse constantly kept as guide. Slowness of pulse can, as a rule, be considered a signum bonum.

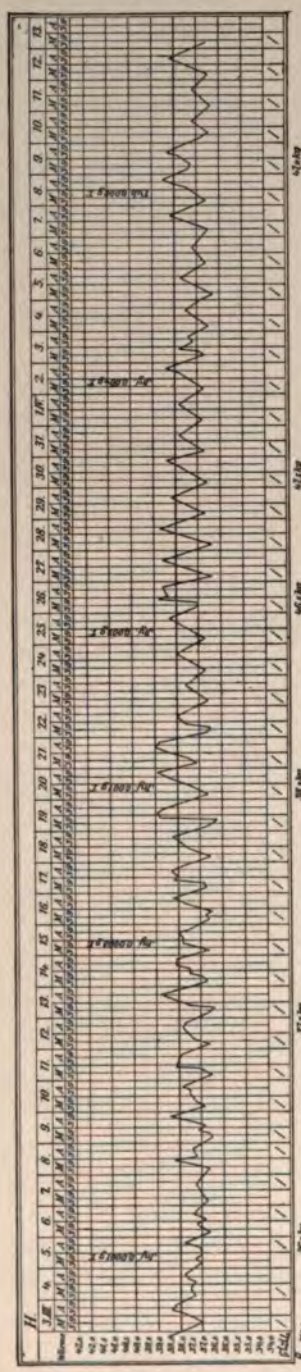

CHART 3.—Striking increase of weight in spite of continuous temperatures.

Especially favorable for the tuberculin treatment are the individuals with a beginning, localized pulmonary tuberculosis, or cases of lupus, and renal tuberculosis, as reported by Lenhartz. The presence of fever leads some to consider such application as contraindicated. This is indeed incorrect, as frequently it is observed that a chronic fever entirely disappears during a course of treatment, and very often remains away. Even if the fever continues, a good result in the general condition of the patient is nevertheless obtained. (Chart 3.)

Patient H.—Ninteen years old with distinct tuberculous habitus, on admission to the medical service, presented a marked infiltration and catarrh of two-thirds of the right lung with a cavity in the upper lobe; infiltration of the left lobe and a great number of tubercle bacilli in the sputum; marked weakness and continuous fever. In five weeks the patient had gained 11 kg. in weight;—8 kg. in one week.

Simultaneously his general condition improved very much; the night sweats disappeared, and the cough diminished, but the number of bacilli still remained the same, and the physical signs of the lungs unaltered. Subsequently the patient received treatment also with B.E., with the consequence that the temperature finally subsided, the cough and sputum likewise, and the bacilli became few and at times entirely absent for several days in succession. In fact, the general condition became excellent. Objectively, there was no demonstration of catarrhal affection.

In this connection it might be noted that such a remarkable increase in weight in so short a time is by no means the rule, although good effects are observed in many cases.

Naturally the medical treatment should not be limited to the tuberculin therapy. If even in the immunization of healthy animals, attention is paid to their housing and feeding, how much more imperative is this consideration when applied to sick human individuals.

Bearing in mind that just as in any other treatment, success is only achieved by creating a favorable medium, the same focussing of good influences should be employed in tuberculin therapy. Rest and forced feeding are curative factors which one cannot neglect, and the best places for the obtention of these, at the beginning at least, are hospitals, sanatoriums or convalescent homes. When in such a way, the general status of the patient is improved, ambulant therapy can be continued.

Contraindications to Tuberculin Therapy.

As for the contraindications to tuberculin treatment, it is very difficult to set general rules. The opinions of various authorities differ greatly on the subject. While for example, Möller and others consider hemoptosis as a distinct contraindication, Aufrecht and Krämer claim that under tuberculin therapy hemoptosis is decidedly improved. It is easy to understand this difference in attitude, if the changes in the focal reaction are considered. There is no doubt that hemoptosis may be excited by increased supply of blood and the inflammatory process associated with the inoculation of tuberculin. The more severe the focal reaction, the greater is this possibility. On the other hand, the new formation of connective tissue and the absorption of the tuberculous tissue will diminish the frequency of hemorrhage. With a general tendency toward hemoptosis, it is therefore best to wait a long time after the cessation of the latter, and then begin with small doses. The patient should be under careful observation and by constant physical examination, any possible focal reaction should be controlled. If, in spite of this, hemoptosis does set in, one should not at once be discouraged. An interval of about fourteen days is to be allowed to pass, and then the treatment again undertaken. Frequently, the hemoptosis will cease. If not, or if the patient loses in weight and becomes weaker, the tuberculin therapy should be discontinued.

As further contraindications, Möller mentions marked general weakness, fever, heart affections, epilepsy, and hysteria. In full agreement with Bandelier and Rœpke, the author does not consider any of the above as cause for the non-employment of tuberculin. Only where absolute cachexia, without any possibility for improvement exists, is this therapy to be omitted. In all other conditions, an attempt is by all means justified. Experience, as a matter of course, plays an important rôle in the selection of suitable cases. For a beginner, it is advisable to gain practice by the treatment of uncomplicated cases before undertaking those of greater difficulty.

2. New Tuberculin, Bacilli-Emulsion (B. E.) and New Tuberculin T. R.

Treatment with new tuberculin follows along the very same lines set down for old tuberculin.

New tuberculin T. R. is the mildest of all preparations. It is very suitable for the beginning treatment of susceptible patients. When the individual does not react to large doses, it is well to start in with B. E. The

employment of B. E. can also be affected without producing any reaction, although this is somewhat more difficult.

The dosage scheme advised by Bandelier and Rœpke is as follows:

1/1000, 2/1000, 3/1000, 7/1000, 10/1000 mg.,
15/1000, 2/100, 3/100, 5/100, 7/100, 10/100 mg.,
At intervals of 1 to 2 days;
15/100, 2/10, 3/10, 5/10, 7/10, 10/10 mg.,
At intervals of 2 to 3 days;
12/10, 15/10, 2, 2 1/2, 3, mg.,
At intervals of 3 to 4 days;
4, 5, 6, 7, 8, 9, 10 mg.,
at 4 to 6 to 10 days intervals.

In susceptible patients, it is best to increase the dosage only by one-half mg. even when large doses are administered. Ten mg. *B. E.* represents the maximal dose.

The author himself follows a different scheme from that of Bandelier and Rœpke. The injections are given less frequently, only about once a week, but the dose is always increased twofold, fivefold and even tenfold without any excessive reactions.

Fever is obtained much less often with new tuberculin than with T. The reaction usually is in the form of lassitude, nausea, weakness, insomnia, etc.

The treatment with new tuberculin is particularly favorable in cases where a low continuous fever is present. It also is more potent in destroying the bacilli of the sputum. The author therefore prefers this, especially the *B. E.* to all other tuberculin preparations. Bandelier and Rœpke have also obtained gratifying results with the *B. E. therapy* as is evident from the following statistics of 205 patients treated at the sanatorium at Kottbus.

Stage.		I.	II.	III.
Cured (A)	23 = 11.22%	10 = 37.04%	13 = 10.48%	0 = 0 %
Completely able bodied (BI)	98 = 47.80%	12 = 44.44%	77 = 62.09%	9 = 16.63%
Satisfactory result (A + BI)	121 = 59.02%	22 = 81.48%	90 = 72.57%	9 = 16.63%
Able bodied in terms of law (BII).	70 = 34.15%	5 = 18.52%	30 = 24.19%	35 = 64.81%
Total improvement (A + BI + BII).	191 = 93.17%	27 = 100 %	120 = 96.76%	44 = 81.44%
Negative result (C)	14 = 6.83%	.....	4 = 3.24%	10 = 18.56%
Bacilli in sputum on admission.	114 = 55.61%	4 = 14.81%	63 = 50.81%	47 = 87.04%
Absence of bacilli or sputum at discharge.	59 = 51.78%	4 = 100 %	49 = 77.78%	16 = 34.34%

In respect to the objection frequently advanced that B. E. is absorbed with difficulty and tends to produce infiltrations, it may be remarked, that this may be readily overcome by preliminary use of T. R. or by the employment of sensitized B. E., as has been advised by Meyer and Rupple.

By sensitized B. E. is understood, a bacilli emulsion which has been mixed with the tuberculous serum of a horse or ox containing anti-tuberculin. This mixture brings about a union between certain of the antibodies and substances contained within the bacteria. The tuberculous serum is then removed by centrifugalization and washing of the mixture with physiological salt solution.

The sensitized B. E. (S. B. E.) is milder than B. E. and in its character is otherwise more like *T. R.* The infiltration is much less, or entirely absent, due, as a general rule, to the fact that sensitization of bacteria tends to neutralize those substances which produce infiltrations. This last has been demonstrated by the author in cases of mouse-typhoid, and swine pest bacilli, where marked infiltrations following their inoculation have by afore-mentioned means of sensitization been avoided. The following chart illustrates preliminary treatment with S. B. E. followed by B. E. (Chart 4.)

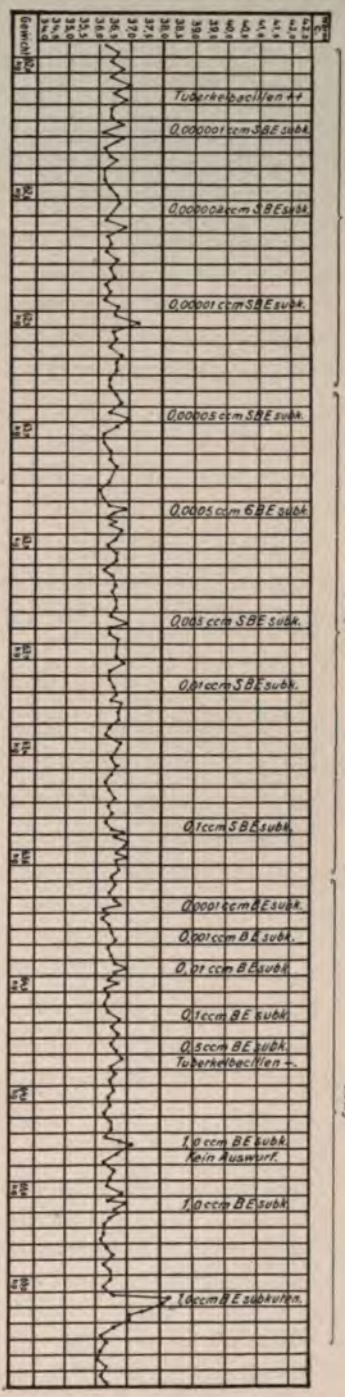

CHART 4.—E. H. 41 years. Intestinal tuberculosis and a double sided pulmonary tuberculosis. Treatment with S. B. E.; almost without any reaction. Immunization with B. E. Relative cure. Disappearance of cough, expectoration, bacilli, catarrhal, symptoms of lung and intestinal symptoms. Increase in weight.

The patient was a female who at the time of admission presented double-sided apical pulmonary and suspicious intestinal tuberculosis. Tubercle bacilli were present in the sputum. The patient was discharged from the clinic as relatively cured, *i.e.*, all manifestations of illness had disappeared with the exception of slight dulness over one of the apices which, however, could have been attributed to cicatrization.

In addition, there was normal vesicular breathing, no temperature, no catarrh, and a good general condition. Undoubtedly it is difficult to say whether this case is cured. Only years of observation can prove this. A temporary latency of symptoms must always be considered. Suffice it to say, that the patient was a great deal improved and able to return to her work.

On close observation of this chart it will be noticed that practically no reactions occurred in spite of the rather rapid increase in the dosage. Slightly increased temperatures were manifest only occasionally (0.00001 c.c. S. B. E.). Even though the same dose was not repeated on account of the long intervals between the individual injections, no increased reaction appeared after subsequent inoculation. When 0.1 c.c. of *S. B. E.* produced no reaction, the susceptibility to B. E. was tested. Doses of 0.0001 B. E. to 0.5 B. E. were administered at short intervals, without any symptoms. Only after 1.0 c.c. of B. E., was there a slight increase of temperature with rather marked general manifestations—(headache, pains in the extremities, weakness etc.) which subsided within twenty-four hours. On repetition of the same dose, no reaction occurred.

Six days later, when for the third time 1 c.c. of B. E. was given there appeared a very marked disturbance throughout the system, an evidence of hyper-susceptibility. (See Chart 2).

3. Bovine-tuberculin.

Koch's differentiation between bovine and human tuberculosis led to the attempt at immunization of cattle with human tubercle bacilli. (Bovo-vaccine of Behring, and Tauruman of Koch).

Spengler tried to reverse this use and employ the milder, infectious bovine bacilli for the tuberculin therapy in man. He used these bacteria to make up preparations similar to the *old and new tuberculin.* In this connection he had recourse especially to the P. T. O. (Perlsucht Original Tuberculin) *i.e.,* the preparation analogous to T. O. A.

Bovine tuberculin is said to be borne better than the human. The reactions are supposed to be of a nature less severe, and the therapeutic results just as good or even better.

4. Nastin.

All the above mentioned preparations have as their aim the production of an inoculation material which is to contain the substances embodied within the tubercle bacilli, and which are more or less correctly considered as representing their poisonous elements. Deycke and Reschad showed that the fat-like material encapsulating the bacteria to which is ascribed their strongly acid fast character, also plays an important rôle in the question of tuberculosis immunity. These men prepared a wax-like substance, nastin, from a streptothrix which they found as a saprophyte in a case of leprosy—streptothrix leproides. Nastin closely resembles the fat-like substance of the tubercle bacilli and *with* it one can immunize healthy guinea-pigs against living virulent tubercle bacilli.

In the treatment of tuberculosis, however, it has no beneficial effect. On the day after inoculation, fever sets in, sputum increases in great quantities and contains large amounts of tubercle bacilli. In leprosy, slight improvement has been noticed by its use.

Metalnikoff has confirmed the above findings and further shown that the bee moth, Galeria Molinella, attributes its very high immunity against tuberculosis to a strong wax dissolving ferment possessed by it. It is probable, too, that inoculations of nastin therefore, produce antibodies which have the power of dissolving fat. In this way the capsule of the tubercle bacillus is destroyed and the antigen is liberated to be absorbed. While healthy animals can thus be immunized, tuberculous individuals would be indirectly receiving a tuberculin injection, and its amount would depend upon the quantity of tubercle substance suddenly liberated. It seems to the author that the more rational way of conducting this therapy would be, to first obtain a high immunity against the substance of the tubercle bacillus by injection with B.E., and then to follow this by treatment with nastin. Such "fractional" treatment may prove an interesting new step in tuberculin therapy.

CHAPTER VII.

Toxin and Antitoxin.

So far, the preceding chapters have dealt with immunization by the bacterial bodies and substances extracted from them. Further attention must, however, be paid to the products of secretion of bacteria, namely the toxins. Only few classes of bacteria have true soluble toxins such as are possessed by tetanus and diphtheria bacilli. The symptom-complex incited by the toxin producing bacteria differs decidedly from that of the sepsis class.

A comparison between anthrax and tetanus certainly exhibits a striking difference. Although both are wound infections incited by characteristic bacteria, smears of the pus from wounds, in the case of anthrax, display on examination numerous bacilli, while in the case of tetanus, the bacillus is very sparsely found. Even carefully prepared anerobic cultures, or inoculations in mice of the pus itself, do not always successfully demonstrate the presence of the tetanus bacillus. In the blood, lymph glands and viscera of anthrax cases, excessively large numbers of microbes can be exposed, while even in the most fatal cases of tetanus, there is nowhere any evidence of bacteria or their spores. Where so many living foreign organisms are found invading the individual, no hypotheses are necessary for explanation of the associated marked disturbances as in anthrax; it is, however, more complex to understand the severity of the symptoms in conditions like tetanus, where such exceedingly scant bacteriological findings exist. Here the micro-organisms play only a secondary rôle, the entire symptom-complex being produced by a poison extruded from the bacteria. In diphtheria, conditions are similar to those in tetanus, although in the former the bacilli can be readily demonstrated both microscopically and by culture. Even though, however, the localization of bacteria in diphtheria is confined to organs not absolutely essential for life—diseased tonsils—these themselves do not explain the alarming situation observed in this disease; for the real cause of the illness is to be found in the toxin which is secreted by the bacteria localized in them, and distributed by the blood stream throughout the entire system.

That a toxin really exists, and is not hypothetical, Roux and Yersin, as *well* as Kitasato have proven by demonstration of the poisonous agents in

the bouillon culture of both diphtheria and tetanus. As most cultures show only slight tendencies to toxin formation, the evidence of a virulent toxin may necessitate a special strain of the bacterium.

The length of time required by cultures for the production of moderate amounts of toxins is by no means constant. With diphtheria this varies from several days to 2 to 3 weeks. As a general rule, if toxin is not liberated within the first four weeks it will most probably not appear after that time. On its appearance, it is isolated by filtering the bouillon culture first through filter paper to remove the pellicle, and then through a bacterial filter to get rid of the bacteria. A layer of toluol 1 to 2 cm. is added for the purposes of sterilization and it is advisable to agitate the toxin and toluol thoroughly every day to prevent contamination.

It does not fall within the scope of this book to take up the various methods proposed for obtaining and preserving the various toxins. It is the object merely to review the details associated with their mode of action and standardization.

The first and most important member of this group is the diphtheria toxin.

Action of Diphtheria Toxin.

The diphtheria toxin is first tested by subcutaneous injections into guinea-pigs 250 mg. in weight. The action of the toxin is entirely dependent upon the dosage; the more toxin injected the more rapidly does death occur. This, however, is not to be taken in mathematically correct proportions—*i.e.*, twice the dose does not produce the same action in one-half the time. *A certain period of time must always elapse before death can take place, the minimum being about one day.* The interim is known as the period of incubation and it is the existence of this that goes to make one of the essential characteristics of a true toxin. A toxin requires a definite period of time for its action to become manifest; and even the largest dose of toxin *cannot diminish the length of this period below a certain minimum.* On the other hand, the length of the incubation time can be increased by the injection of a smaller dose, so that ultimately a dose small enough is obtained which is not instrumental in producing death (Dosis subletalis).

If a guinea-pig is inoculated with a quantity of toxin sufficient to kill it in three to four days, nothing abnormal is evident the first day; various manifestations of illness, however, follow soon after.

Edema appears at the site of inoculation. The animal stops eating, sits in a corner, and reacts poorly to sound. Gradually it becomes weaker, so that when placed upon its back it does not resume its normal position; the temperature which at first rose somewhat, falls abruptly and then death takes place.

At autopsy, a gelatinous and strongly hemorrhagic edema is found which starts at the site of the injection. On opening the abdominal cavity one finds but very little peritoneal exudate, strongly injected vessels of the mesentery, and especially characteristic, markedly reddened adrenal glands. In the thorax are found bloody pericardial and pleural exudates, and consolidated areas in the lungs.

After the injection of smaller doses, edema likewise arises and becomes larger in extent the slower the case progresses. Besides this, the animal loses in weight. With sublethal doses, edema or infiltration is confined to the site of injection, and finally, with the minutest doses, no edema occurs, but the hair falls out at the place of injection.

Guinea-pigs surviving a dose of toxin, may after two to four weeks begin to show paresis first of the hind, then of the fore extremities, and finally even of the muscles of the back and respiration. The most severe types of such conditions, however, may fully subside. They may be considered as analogous to the post-diphtheritic paralysis taking place in man, which is usually, as is well known, of a benign nature.

Besides guinea-pigs other animals suitable for diphtheria experimental work are rabbits (especially by intravenous injection) and pigeons (by intramuscular injection).

The susceptibility of animals towards diphtheria toxin varies greatly, as is seen from the following scale of Behring, the least susceptible animals being mentioned first: mouse, rat, dog, guinea-pig, rabbit, sheep, cow, horse, goat.

Estimation of Strength of Diphtheria Toxin.

The strength of the diphtheria toxin is estimated as follows: Guinea-pigs of equal size (250 gms.) receive subcutaneous injections of decreasing amounts of toxin. With a strong toxin, centi- and milligrams or even smaller denominations are of sufficient potency to produce death. Doses such as these are not injected unless diluted in normal salt solution. For exact results one must not depend upon the findings brought out by the injection of a single animal with each dilution; several should be inoculated with the same dose and the effects, which should be the same in all cases, noted. It is impossible to state beforehand how many dilutions may be necessary. If the various actions, dependent upon the successive gradations of dosage are successfully represented, the experiment may be taken as conclusive; that is to say, the smallest doses must leave the animal entirely unaffected, the moderate produce slight local and general symptoms, and the larger ones cause death of the animals. If it should so happen that they all die, a new set of experiments employing a lower scale of dosage should be undertaken.

Thus it is seen that the *action of diphtheria toxin is subject to the quantity of the toxin injected.* If several different diphtheria toxins are tested at the same time, it is at once evident what far reaching differences may arise. While 0.001 c.c. of one diphtheria toxin kills a guinea-pig in twenty-four hours, a different diphtheria toxin performs the same action with a dose ten times as great, *e.g.*, 0.01 c.c. The second toxin thus contains only one-tenth as many of the active substances. *In order to obtain a uniform method for estimating the strength of a diphtheria toxin and thus get comparative values, a standard unit of the same has been adopted. And this consists of the smallest amount of toxin that will kill a healthy guinea-pig weighing about 250 gms. in four to five days.* This is known as the *minimum lethal dose or dosis letalis minima.* In addition to this "direct toxic value," it is frequently important, especially for the standardization of curative sera, to estimate the "indirect toxic value" by which is meant the amount of antitoxin which a toxin can bind or neutralize.

Active Immunization against a Toxin.

If an animal, *e.g.*, a goat is injected with a sublethal dose of diphtheria toxin and after the lapse of a certain period of time it is reinjected with a lethal dose, the animal remains alive. In fact it may receive numerous fatal doses, and still survive. This experiment is the simplest in active immunization against a toxin. The explanation of the action which has taken place, an examination of the blood serum of the immunized animal will disclose very readily. If this serum is mixed with a fatal dose of toxin and the mixture inoculated into a normal guinea-pig, the latter remains alive and perfectly active. The serum of the immunized animal therefore contains a protective agent which is directed against the toxin and destroys its activity; hence the name antitoxin. But *the antitoxin is specific, i.e.*, diphtheria antitoxin neutralizes only diphtheria toxin and not tetanus. The recognition of these facts and those heretofore mentioned, and the recommendation of the therapeutic use of diphtheria serum belongs entirely to v. Behring and righteously may he be called the father of serum therapy.

Antitoxin.

Although theoretically, the serum of any animal immunized with diphtheria toxin can serve as a curative serum for diphtheria, practical experience has taught that it is best to employ horses for this purpose. For laboratory experiments goats should be the animals of choice. It is advisable to use the above animals for the reason that larger quantities of serum are obtained and furthermore because *it has been found impossible to immunize guinea-pigs with previously unchanged diphtheria toxin even if the initial dosage used is the smallest subdivision of the minimal lethal dose.* Behring and Kitashima showed that after repeated injections of very minute doses they were able to kill guinea-pigs even with 1/400 of the dosis letalis minima. This is but another example of an effect just opposite to that of immunity and known as *hypersusceptibility* or *hypersensitiveness*, which has already been described in the chapter on tuberculin therapy. If, however, it is desired to immunize guinea-pigs, a *modified form of the diphtheria toxin* must be employed for the first injections. Several modifications are feasible. Behring and Kitasato added iodin trichlorid to the toxin while Roux and Martin, Lugol's solution; C. Fränkel heated it to 60°, and Behring advocated the so-called "simultaneous method" (of special aid in tetanus toxin), where mixtures of toxin and antitoxin are injected and gradually the quotient of the latter is diminished until finally it is entirely omitted. If the animals have borne the first inoculations of the modified toxin without any ill effects, one may then devote himself to the use of the unmodified toxin.

In contrast to small animals, horses can be immunized with unmodified diphtheria toxin right from the start. Nevertheless great care must here also be exercised. Certain it is, that less risk is run in the employment, with even the larger animals, of a modified toxin. For the production of a good diphtheria serum, healthy horses about five to six years old are used and into them gradually increasing amounts of diphtheria toxin are injected subcutaneously or even intravenously; thus agreeing with Ehrlich's findings to the effect that the antitoxin content of a serum can be raised by successively increasing the amount of toxin injected. As far as the efficiency of the serum produced is concerned, it is entirely dependent upon the animal. Horses vary greatly in their individual predisposition towards the making of an effective serum; some animals even completely

fail to do so, not that the latter are not actively immunized, for they are, but because they contain very little antitoxin within their serum.

It is impossible to recommend a distinct scheme for the immunization of a horse. The intervals between the injections and the size of the dose is entirely dependent upon the reaction of the animal toward previous inoculations. *A good rule to follow is, that a fresh injection should be given only if the reaction from the preceding one has entirely subsided. The reactions are both local and general.* The local reaction comes in the form of edema, infiltration, and sterile abscesses; the general, loss in weight and appetite and increase in temperature.

The following chart of Salomonsen and Madsen, of the Copenhagen Serum Institute, serves as an example of how such a diphtheria serum is produced. A gravid mare 665 kg. in weight was selected and injections were given as follows.

Day.	Dose of Toxin.	Remarks.	Day.	Dose of Toxin.	Remarks.
1	1 cm.		135	.....	Serum removed contained 150 immunity units.
6	1 cm.				
12	3 cm.		154	.....	Birth of colt.
15	5 cm.		177	.....	Serum removed contained 45 immunity units.
23	10 cm.				
27	20 cm.		184	100 cm.	
36	25 cm.		188	200 cm.	
41	50 cm.		195	400 cm.	
45	75 cm.		205	700 cm.	
50	100 cm.		213	800 cm.	
57	150 cm.		223	600 cm.	
72	250 cm.		232	600 cm.	
81	450 cm.		242	1000 cm.	
92	600 cm.		252	.....	Serum removed contained 120 immunity units.
104	800 cm.				
119	1000 cm.				

The time selected for venesection is important. Antitoxins like any other antibodies do not arise immediately after an injection, but only after a certain *incubation period.* The amount of antitoxin first gradually increases, then begins to sink, and after that remains constant for a certain period until it finally disappears. If at a time when the serum contains a certain amount of antitoxin a new inoculation is undertaken, the so-called "negative phase," sets in, *i.e.*, the amount of antitoxin within the serum first sinks and then is followed by a compensatory rise. By becoming acquainted with the wave-like fluctuations in the antitoxin content of the serum, and renewing the injection at the time of highest content, one can produce a serum with

very strong antitoxic qualities. This was done by Salomonsen and Madsen who by experimentation found that the maximum height of the antitoxic curve was reached on the tenth day after each inoculation. For this reason it is wise to choose this day for the removal of the serum. As regards other sera, *e.g.*, tetanus, different periods have been empirically found to be most serviceable. As the antitoxic curve does not remain at a high point for a long time, the injections should be repeated from time to time. For highly immunized horses, monthly injections usually suffice.

After the serum has been obtained, the important problem which arises is how to keep it sterile. This is accomplished by aseptic precautions at the time of the obtention of the serum and eventually by the addition of preservatives such as 1/2 per cent. carbolic.

This procedure finished, the next step is to estimate the amount of the antitoxin content in the serum.

According to v. Behring and Boer, the value of the serum should be ascertained in respect to its:

1. Protective power } against infection.
2. Curative power }
3. Protective power } against intoxication.
4. Curative power }

v. Behring found that these four properties run parallel with each other so that for practical purposes, it suffices to establish only one of these qualities. For diphtheria serum it has proved most serviceable to estimate the strength of the immunity against intoxication, since one is dealing with a purely antitoxic serum.

Standardization of Diphtheria Serum.

Behring's original mode of standardization consisted in gradually adding doses of serum to the minimal lethal dose of toxin and injecting the mixtures into guinea-pigs, thus determining the smallest amount of serum capable of preventing death of the animal. It was soon found, however, that this method gave too inconstant results because the individual minimal lethal dose was too variable.

Ehrlich, therefore, modified the process by using ten times the minimum lethal dose. This amount of toxin, mixed with decreasing amounts of serum and made up to 4 c.c. with physiological salt solution was injected subcutaneously into a guinea-pig. The smallest amount of serum which saved it from being killed on the fourth to fifth day was thus estimated.

The method of standardization used at the present time owes its origin to Ehrlich.

In order to attain uniformity in the comparative value of all sera, Behring and Ehrlich recommended the adoption of two empirical values; "the normal toxin," and the "normal curative serum."

The normal diphtheria toxin is one which contains enough toxin in 1 c.c. to kill 25,000 gm. of guinea-pigs or 100 guinea-pigs each weighing 250 gm.

A normal curative serum is one of which 0.1 c.c. suffices to neutralize

1 c.c. of Behring's normal poison, *i.e.*, is able to overcome the effect of 100 fatal doses. 1 c.c. of this normal curative serum represents one immunity or antitoxin unit.

The present antitoxin unit was fixed by Ehrlich who adopted that amount of antitoxin as his standard, which when mixed with 100 times the lethal dose of a then existing toxin, and injected into an animal, was sufficient to so neutralize the toxin that not the slightest evidence of either a local symptom or general illness was present. Ehrlich chose the antitoxin rather than the toxin as the constant of standardization, because the toxin would deteriorate after some time, while the antitoxin could be preserved in a stable, unchangeable form.

In spite of this fact, the new method of titration was still unsatisfactory, inasmuch as the toxin could undergo other biological changes not yet taken into account.—To understand these, the acquaintance of several new terms is essential, and they are, *dosis certe efficax, limes + or limes death, limes o or limes zero.*

While the dosis letalis minima represents the smallest dose of toxin which may be fatal in four to five days, the dosis certe efficax (dose of certain efficiency) stands for the smallest dose which will surely kill any pig of 250 gm. within this period of time.

By limes + (limes death) is meant the smallest amount of toxin which after being mixed with an antitoxin unit, will still cause the death of a guinea-pig within four to five days. By limes o (limes zero) is understood the dose of toxin which is just neutralized by one antitoxin unit (I. E. = antitoxin unit or "Immunitäts Einheit"), so that no toxin is free and the animal remains perfectly well. Limes + therefore implies an excess of poisonous toxin; L O, perfect neutralization.

Theoretically speaking, the difference between L + and L O should represent the minimum lethal dose (d. l. m.). This, however, is almost never so, as is shown in the following illustration.

The d. l. m. of a certain poison was estimated as 0.0039 c.c.

L+ was found to be 0.48 c.c. = 123 lethal doses.

LO was found to be 0.42 c.c. = 108 lethal doses.

Difference 0.06 cm. = 15 lethal doses.

In order to explain this phenomenon Ehrlich considered that there were two other substances contained within the diphtheria bouillon in addition to the diphtheria toxin; namely, diphtheria toxon and diphtheria toxoid.

Toxon. The toxon is a poison which in contrast to the toxin has only a slight affinity for the antitoxin. It is this body which is probably the cause of the paralysis occurring weeks after the infection.

In a mixture like L O, the antitoxin has fully neutralized both the toxon as well as the toxin. If, however, more diphtheria poison is added to the

I. E., as is done in the L +, the antitoxin, on account of its greater attraction for the toxin, will combine with the latter and leave the toxon free to subsequently carry out its own functions. The more crude poison is added, the more toxon remains unbound, until a point is reached when no more toxin can be taken up and consequently some is left unneutralized. If the amount of active toxin reaches the dosis letalis minima, it is sufficient to kill the animal and thus the limes + is attained.

When, instead of freshly prepared toxins Ehrlich employed older bouillon cultures, the poisonous qualities distinctly sank to about one-half, but the surprising fact was that the L + had not been altered and even though it had lost one-half of its toxic power, it had still retained its initial potentialities for neutralizing antitoxin.

Toxoid.

Ehrlich's explanation was that the diphtheria poison consists of two molecular groups; one the carrier of the toxic qualities, and therefore known as the "toxophore" group, the other uniting with the antitoxin and having the capability of neutralizing it, known as the "haptophore group." The toxophore group is very labile, while the haptophore group strongly in contrast to it, is characterized by its stability. The toxophore element destroyed, the diphtheria poison loses its toxic qualities, but retains its power to bind antitoxin. A non-poisonous diphtheria toxin possessing such power, is designated by Ehrlich as "Diphtheria Toxoid."

The mode of standardization of serum advocated at the present day is applicable exclusively to the L + dose. It is effected by injecting guinea-pigs subcutaneously with mixtures of various doses of diphtheria toxin on hand, plus an anti-toxin unit, and noting the smallest amount of toxin which kills the animal in four to five days. This L + as the constant factor is now mixed with different amounts of the serum to be tested and that quantity determined which just prevents the death of the animal. If for example 1/100 c.c. is necessary, this serum is considered one hundred times as strong as the standard antitoxin unit, or in other words it contains 100 immunity units.

This method of Ehrlich has been adopted not only in Germany, but almost in all other countries in Europe and also in America. Only in France the principle varies somewhat, as here the serum is tested both for its protective and curative action. The *protective power* of a serum is considered 50,000 if 0.01 c.c. of a serum saves a guinea-pig weighing 500 gm. from the fatal consequences following a dose of toxin sufficient to kill an animal of the same weight in thirty to forty hours. The standard therefore takes into consideration the relation between the amount of serum and the weight of the animal. The serum is injected into the guinea-pig twelve hours before the toxin and the animal should not lose in weight during the following six days. The *curative power* is estimated by injecting a guinea-pig with a dose of toxin (sufficient to kill a control animal in thirty to forty hours) and six hours afterward the serum is injected. The animals remaining alive on the sixth day are considered as cured.

The French method of standardization is built upon the belief of Roux that no parallelism necessarily exists between the protective and curative values of a serum. Kraus and Schwarz have recently published accounts

of experiments which corroborate Roux's views. They claim that a very highly valent diphtheria serum has a lower curative value than one less so; that the curative power of a serum does not depend upon the increase or decrease of the antitoxin content during the immunization of an animal, and that Ehrlich's process of standardization taking into consideration only the protective power, requires additional modification. Berghaus working in Ehrlich's Institute answered the above exceptions in so satisfactory a manner, that up to the present day Ehrlich's views are still upheld by the majority of workers in this field.

While in some countries the government institutes have complete control over the production of diphtheria serum, in Germany it is manufactured by private concerns, but under government supervision.

The serum must be absolutely clear, free of bacteria and toxins, especially tetanus toxin, and must not contain more than 1/2 per cent. of phenol. It should contain at least the number of antitoxin units designated by the factory.

In the United States the standard antitoxin is distributed by the Public Health and Marine Hospital Service Laboratories. Since 1902 the production and sale of diphtheria antitoxin has been regulated by law.

At frequent intervals, antitoxin is bought in the open market and examined at the hygienic laboratories of the United States Public Health and Marine Hospital Service. Antitoxic serum containing less than a hundred units to each cubic centimeter is precluded from sale.

The Serum Therapy of Diphtheria.

In man the antitoxic diphtheria serum is used with success for both curative and prophylactic purposes.

For therapeutic application it is of the greatest importance to employ the serum in sufficient quantities and as soon as possible. The value of early intervention can be seen from the following chart of Kossel:

Day of illness.	Treated.	Cured.	Died.	Percentage of cures.
1	7	7	0	100
2	71	69	2	97
3	30	26	4	87
4	39	30	9	77
5	25	15	10	60
6	17	9	8	47
7–14	41	21	20	51
Indefinite	3	2	1	.....
	233	179	54	77

Large doses of antitoxin should be administered right from the start. The old practice, still employed by few, of using small doses is to be condemned,

for the aim in the treatment is to neutralize as soon as possible all the free and partly bound toxin.

According to the researches of Doenitz, more recently confirmed and extended by Fritz Meyer, it was established that large amounts of antitoxin can even neutralize toxin already attached to the tissue cells. Men with practical experience like Heubner, give 4000 units as the initial dose. In the United States doses as high as 10,000 to 100,000 I. E. have been administered with good results. The view of large dosage is being gradually taken up also in Germany. At any rate it is by far better to give too much than too little. If the first injection does not suffice it should be repeated the next day. The only possible drawback associated with the use of excessive amounts is the possibility of *serum sickness*, to be mentioned later. Netter has found that the administration of 1 gm. of calcium chloride on three successive days prevents serum sickness.

The serum has thus far been as a rule injected subcutaneously. This method is very practical and as far as anaphylaxis is concerned, is the least dangerous. The disadvantage, however, is that it is very slowly absorbed. Madsen and Hendersen-Smith have shown that but a trace of antitoxin can be found in the blood of the patient four and three-fourth hours after the injection, and only after two to three days can larger amounts be demonstrated. In view of this, Morgenroth recommends *the gluteal intramuscular injection* for here a much more rapid absorption follows. In cases of dangerous illness intravenous injection may be undertaken. For this purpose Meyer advises a serum free of carbolic acid, although this is not absolutely necessary.

The importance of the method of injection is clearly shown by the comparative experiments of Berghaus. In order to save a guinea-pig injected with a definite amount of toxin and followed in 1 hour by antitoxin, it was necessary to employ:

0.08 I. E. by intracardial injection.
7.0 I.E. by intraperitoneal injection.
40.00 I. E. by subcutaneous injection.

Thus the curative power was increased 500 fold by placing the antitoxin directly into the circulation.

The treatment of diphtheria must by no means be limited to serum therapy. A symptom of grave prognosis is the lowered blood pressure which must be counteracted by infusions of 1/2 liter of physiological salt solution containing five to six drops of adrenalin.

The question whether the use of concentrated antitoxin is therapeutically more efficient than the non-concentrated is still a matter for discussion. Numerous authors claim that sera of medium strengths (about 400 I. E.) are most efficient. The highly concentrated sera are much more expensive.

For *prophylactic purposes* 500 to 1,000 units injected subcutaneously usually suffice. Protection thus attained lasts about three weeks.

CHAPTER VIII.

Toxin and Antitoxin (*continued*).

Definition of Toxin, Tetanus Toxin, Botulism Toxin, Dysentery Toxin, Staphylolysin.

The diphtheria toxin and its antitoxin just discussed in detail is of great practical and theoretical importance, and can serve as a type of all true toxins and antitoxins. *Bacterial toxins can be defined as poisons given off by the bacteria, the symptoms resulting from their action appearing after a certain incubation period. The invaded organism reacts by the production of specific antitoxins which neutralize the toxins in amounts, following the law of multiple proportions.*

Further analysis of this definition indicates that a substance can be considered a toxin only when it has a poisonous action, or in the words of Ehrlich when it possesses a toxophore group.

This toxicity does not always manifest itself by necrosis or death as in diphtheria. More frequently the toxin has a somewhat selective action affecting a certain group of organs. Thus a toxin acting upon the central nervous system or blood is designated respectively as a neurotoxin or a hemotoxin. To differentiate a true toxin from other poisonous products obtained from bacteria, it is important to note that all true toxins are elements of *secretion* of the living bacteria, and can be separated from them by filtration. According to this definition poisons contained within the bacterial bodies themselves, which may be liberated by various mechanical, physical, or chemical means, cannot be considered as belonging to the class of true toxins. These poisons are characterized by peculiar properties and are known as *endotoxins*. In addition it may be remarked that inasmuch as a true toxin requires, a period of incubation in order to manifest its action, those toxins which act spontaneously are to be excluded from the former group. R. Krause nevertheless considered some of the poisons isolated from the cholera and cholera-like spirilla (El Tor Vibrio) as true toxins even though they lack an incubation period.

The real essential property of a toxin is doubtlessly that one can immunize against it, and be able to demonstrate the presence of antitoxins within the serum of the immunized animal. Ehrlich furthermore claims that the amount of antitoxin produced follows the law of multiple proportions. By

this is meant that the relationship between a definite dose of toxin and the amount of antitoxin just sufficient to neutralize it is constant, so that if ten volumes of toxin hold in bounds ten volumes of antitoxin, 100 volumes of toxin neutralize 100 volumes of antitoxin. This relation is best exemplified by the diphtheria toxin and antitoxin. With the other toxins, conditions are more complicated so that many objections have been raised against the above rule of multiple proportions. (Bordet, Arrhenius, Madsen, etc.)

The true toxins causing infections in man are limited to the

1. Diphtheria toxin.
2. Tetanus toxin.
3. Botulism toxin.
4. Dysentery toxin.
5. Staphylolysin and similar bacterial hemotoxins.

Tetanus Toxin.

Tetanus Toxin.

The tetanus toxin is found within filtrates of bouillon cultures of the tetanus bacillus. While partial erobiosis does not entirely eliminate toxin formation, anerobic conditions are by far more favorable for it. The tetanus toxin is of two kinds; the tetanospasmin, and tetanolysin; the former a neurotoxin, the latter a hemotoxin. The tetanospasmin is the more important of the two for the reason that it is the agent which produces convulsions. If susceptible animals such as mice or guinea-pigs are injected subcutaneously or intramuscularly with tetanus toxin, after a certain interval—the incubation period—they will begin to show symptoms due to tetanospasmin. They become hypersensitive to reflex stimulation; clonic convulsions and toxic rigidity of the muscles set in. In animals the last state appears first in the group of muscles nearest the point of injection, while in man the spasm almost regularly starts in the muscles of the lower jaw. By intravenous and intraperitoneal injections, the tetanic spasm appears simultaneously in all muscles of the body; on intracerebral inoculation, Roux and Borrel describe the occurrence of epileptiform seizures, polyuria and certain motor disturbances—the entire set of complications being known as cerebral tetanus. Rabbits receiving very small amounts of tetanus toxin intravenously die after gradual emaciation and marked cachexia. This type of infection is designated by Doenitz as tetanus sine tetano. If taken per os, tetanus toxin manifests no poisonous symptoms. Tetanospasmin is a distinct nerve poison especially affecting the central nervous system.

Experiments by Wassermann and Takaki have demonstrated an especially close affinity existing between the tetanus toxin and certain organs. These organs differ in different animals. Thus in man, horse, and guinea-pig only the central nervous system, while in rabbits in addition to this, also the liver and spleen take up the tetanus poison.

If an emulsion of brain tissue and a fatal dose of tetanus toxin are mixed and the mixture injected into mice, the latter remain unaffected. According to Doenitz only the gray matter and not the white substance of the brain possesses this absorption power. If the brain emulsion is boiled, however, it loses this affinity for the toxin.

Concerning the way by which the toxin reaches the central nervous system, opinions vary. Most writers, especially Meyer and Ransom, consider that the journey is made along the nerve paths. Zupnik on the other hand believes that it is distributed through the blood stream and is taken up not only by the nervous system, but also to a great extent by the muscles.

That tetanus toxin is very labile is well known. According to Kitasato, five minutes at 65° C. or twenty minutes at 60° is sufficient to weaken the toxicity to a great extent, in fact even almost to destroy it. Light has a similar effect upon it. Careful as its preservation may be, the soluble tetanus toxin soon becomes attenuated. Hence the best way of keeping it in stock is in a dry form. For estimating the strength of the toxin white mice are employed and are subcutaneously injected with fresh soluble toxin, the lethal dose being the amount which kills the animals in four to five days. Animals more susceptible than mice are horses, they being twelve times as sensitive and guinea-pigs six times as much. Hens possess greater power of resistance, being 30,000 times less susceptible to the toxin than mice.

Tetanolysin acts upon the red blood cells and disintegrates them. The erythrocytes of goats, sheep and horses, are best suited for experiments to demonstrate this action. Ehrlich showed that the tetanolysin and the tetanospasmin are really two identically different toxins and not one toxin with a twofold function. When tetanus poison is mixed with red blood cells the tetanolysin is absorbed and the tetanospasmin remains free. Even the antitoxins of these two are different.

As far as the standardization of the tetanus serum is concerned, it is affected on the same lines as the diphtheria serum, *i.e.*, the L + dose of toxin being the one employed.

"In America the method of standardization was regulated by a law passed in July, 1908, based upon the work of Rosenau and Anderson at the United States Hygienic Laboratories at Washington. Their unit of antitoxin is ten times the smallest amount of serum necessary to save the life of a guinea-pig for ninety-six hours, against the official unit of standard toxin. This toxin unit consists of 100 minimal lethal doses of a precipitated toxin preserved at the hygienic laboratory of the Public Health and Marine Hospital Service. At the hygienic laboratory at Washington a standard toxin and antitoxin are preserved under special conditions, and standard toxin and antitoxin, arbitrary in their first establishment, are kept constant by being measured against each other from time to time. For details of this standardization the original article in the United States Hygienic Laboratory Bulletin 43, 1903, should be consulted."

Serum Therapy of Tetanus.

In regards to the efficiency of serum therapy in tetanus, opinions differ. There is, however, no doubt that a certain amount of reliance can be placed upon this treatment. Failures in successful application are ascribed to the different paths by which the toxin and antitoxin travel. The former is carried along by the nerve fibers, while the latter by the blood stream. Thus the serum instead of being given subcutaneously, as is the general rule, is administered by intraneural, intracerebral, and subdural injections. 100 to 200 units should be injected subcutaneously at the site of the infection or its vicinity and in addition the nerve fibers supplying the infected region should be exposed and inoculated with moderate doses of antitoxin at various points along their centripetal course.

The prophylactic use of tetanus serum has met with better results. Behring advises the administration of ten to twenty antitoxin units subcutaneously. Calmette sprinkles upon the open navel at birth a powder made of dried serum as a prophylactic against tetanus neonatorum. Bockenheimer advises an ointment containing the antitoxin as a dressing for suspicious wounds.

Botulism Toxin.

The Botulism toxin is the poison produced by the bacillus botulinus. This is the exciting agent of a type of meat and sausage poisoning described by van Ermenghem in 1896 as Botulism. The bacillus botulinus is a very actively motile anerobic bacterium which grows at room temperature and presents marked gas and toxin formation. A medium in which the toxin is readily produced consists, according to Ermenghem, of an alkaline bouillon made in the form of an infusion from ham with the addition of 1 per cent. of glucose, 1 per cent. of peptone and 1 per cent. of NaCl.

The toxin can thus be demonstrated after 3 weeks of growth, and is then obtained by bacterial filtration. The cultures have a sour odor like unto butyric acid. The toxin deteriorates easily when exposed to air and light. It is therefore preserved in brown, sealed vials, and kept on ice; or, in a dried form in vacuum. Heating the toxin for three hours at 58° or one-half hour at 80° destroys its toxicity.

Acting unrestrained, the botulism toxin is one of the severest of poisons. It affects susceptible animals even in minutest doses. In contradistinction to other toxins it is fatal even when taken per os.

The characteristic symptoms produced by botulism intoxication consist of hypersecretion of mucus from the mouth and nose, paralysis of eye muscles, urine retention, obstipation, dysphagia, aphagia, and aphoria. No fever, nor any sensatory disturbances are in evidence. Death takes place because of bulbar paralysis accompanied by respiratory and cardiac failure.

The poison is absorbed or arrested in the central nervous system. 0.1 c.c. of an emulsion of central nervous tissue neutralizes three times the fatal

dose for mice. Lecithin, cholesterin, as well as fatty substances like butter and oil, act in a similar manner.

Monkeys, rabbits, guinea-pigs, mice and cats are susceptible to the toxin.

Cats usually exhibit the most characteristic clinical picture. Localized and almost pathognomonic paralyses occur in the form of prolapse of tongue, marked mydriasis, aphonia, aphagia, etc.

In mice, paralysis of the hind extremities sets in after quite a small dose; and death follows in a few hours.

In rabbits and guinea-pigs, moderate doses (0.0003–0.001 c.c.) occasion no manifestations during the first two to three days, but subsequently, the above mentioned paralyses arise and several hours after the animals expire. With larger doses (0.1 to 0.5 c.c.) the incubation period lasts only a couple of hours and then dyspneic attacks usually succeeded by motor paralysis and death are the consequences.

The strength of the botulism toxin is ascertained by injecting guinea-pigs subcutaneously and observing the time when loss in weight, flabbiness of abdominal muscles and death occur.

The following chart by Madsen exhibits the above principle.

Dose in c.c.	Result.	Dose in c.c.	Result.
0.0015	+ on 1st. day	0.0009	Weakness in 3 weeks.
0.0015	+ after 1 1/2 days	0.0009	Loss in weight.
0.0015	+ after 2 days	0.0009	Loss in weight.
0.0013	+ after 2 days	0.0007	Loss in weight in 2 weeks.
0.0013	+ after 5 days	0.0007	Loss in weight in 1 week.
0.0013	+ after 6 days	0.0007	Loss in weight in 1 week.
0.001	+ after 4 days	0.0005	Loss in weight in 1 week.
0.001	+ after 5 days	0.0003	Practically no symptoms; only several days of weakness.
0.001	+ after 5 1/2 days		

Kempner immunized goats against Botulism toxin and proved the presence of antitoxins within their sera. Immunization of rabbits and guinea-pigs is only feasible if primary inoculations are made with a toxin previously attenuated by heat for one-half hour at 60° C.

Recently Wassermann has immunized horses against this toxin. *In view of the high mortality and lack of any other specific medication, the use of this serum is strongly advised.* In animal experimentation it shows itself of undeniable value. As for its effects in man, it has not been employed frequently enough to judge.

The botulism toxin and antitoxin unite only very slowly. Otto and Sachs have shown that the inoculation of rabbits with a three hours old mixture of toxin and antitoxin occasioned greater toxic effects when administered intravenously than when given *subcut*aneously. Only in mixtures twenty-four hours old was this difference overcome.

Dysentery Toxin.

Dysentery toxin was first demonstrated by Conradi. Subsequently from experiments by Rosenthal, Todd, Kraus and Doerr, etc., it became evident that this was a true toxin and not an endotoxin as was originally considered. Only the Kruse-Shiga type of bacillus forms a toxin; for the Flexner kind, no definite toxin has as yet been isolated. Recent investigators, however, especially Kraus and Doerr are inclined to consider the human dysentery of the Kruse-Shiga origin in the light of an intoxication or toxemia similar to diphtheria. The lesions in the large intestine where the bacteria accumulate can be compared to the diseased diphtheria tonsils, while the other manifestations, as the central symptoms, cardiac disturbances, nervous sequelæ, eye affections, etc., can be taken as expressions of the toxemia.

Like the other described toxins, the dysentery toxin can be obtained by filtration of bouillon cultures. The meat infusion must be quite alkaline. The optimum alkalinity, according to Doerr, is obtained by adding 0.3 per cent. soda to litmus neutral bouillon. The precipitate thus formed which increases on sterilization should not be removed by filtration. Doerr also advises finely powdered chalk (20 gm. pro liter) to be added to the weakly alkaline bouillon before the last sterilization. The toxin is formed very gradually; the maximum is derived after two to three weeks. The gray white pellicle upon the surface of the culture can be taken as an indicator for the amount of toxin present.

According to Kraus a good dysentery toxin can also be made by emulsifying the bacteria (grown upon agar) in physiological salt solution and filtering this through Reichel filters.

The toxicity of individual strains of dysentery bacilli varies greatly.

The strength of the toxin is diminished by heating for one to two hours at 60° C. Higher temperatures destroy it, as 80° C. where destruction occurs in three minutes and 90° to 100° C. in one minute.

Acids destroy the toxin probably by the formation of a non-poisonous compound. The addition of a strong alkali restores the toxicity.

Its preservation can be accomplished in a fluid state under the cover of toluol.

The action of dysentery toxin can best be studied by its effect upon rabbits after intravenous inoculation. Large doses kill the animals in very short time, six to seven hours. The ordinary lethal dose produces characteristic symptoms consisting of paresis, diarrhea, which may be bloody, paralysis of the bladder, hypothermia, etc. Death takes place in three to four weeks.

Given subcutaneously, or intraperitoneally, the toxin has only a very mild action. The incubation period is especially prolonged. Given per os, no effect is in evidence.

Besides rabbits the other susceptible animals are monkeys, cats and dogs (to large doses); chickens, pigeons and guinea-pigs are, in the opinion of Kraus and Doerr, not at all affected by the toxin.

The intestinal changes found at post-mortem examination of the animals very closely simulate the pathological alterations occurring in man. A hemorrhagic necrotic enteritis is present which in rabbits is regularly localized in the appendix and cecum, while in dogs

the entire intestinal tract and especially the duodenum is attacked, and in monkeys the lower part of the intestine is involved.

The associated nervous manifestations are, according to experiments of Dopter, referred to changes in the spinal cord itself. These are of a nature similar to acute anterior poliomyelitis. Occasionally a polio-encephalitis is added.

Dysentery Serum.

An antitoxic dysentery serum is obtained by immunization of horses and goats. Various methods have been employed in its obtention. Of the older authors, Shiga and Kruse immunized animals with dysentery bacteria and thus produced a serum which possessed besides its bacteriolytic and agglutinating properties also a weak antitoxic action. Rosenthal, Todd, Kraus and Doerr employed the toxin itself for immunization purposes.

In standardization of the serum the properties to be determined, are three. [Kraus and Doerr employ rabbits in this work.]

1. *Its power of neutralizing toxin in vitro.*—Toxin and antitoxin are mixed in various proportions; the mixtures allowed to stand fifteen minutes at room temperature and then injected intravenously.

2. *Its power of neutralizing toxin in vivo.*—The toxin is injected into the right vein and the antitoxin at the same time into the left vein.

3. *Its curative power.*—The antitoxin is injected at various intervals after the toxin.

These three therapeutic factors do not appear simultaneously. The power of neutralization in vitro is first in evidence. Only very much later does the serum develop its curative strength and ability to neutralize in vivo.

In animal experimentation, the antitoxic serum exhibits its neutralizing and curative properties only in cases where intravenous injections are applied.

Dysentery serum has been employed with fairly good results. Infections caused by the Shiga-Kruse bacilli can, however, alone be benefited. The serum should be given subcutaneously and as early in the stage of the disease as possible. The dose advised by the different authors varies greatly, on account of the inconstancy in strength of the numerous sera and the severity of the infection. In cases of moderate illness, it is as a rule sufficient to give one to two injections of 20 c.c. of a strong antitoxic serum which can neutralize toxin both in vivo and in vitro. Vaillard and Dopter have injected as many as 80 to 100 c.c. in the severer cases.

The good effect of the serum manifests itself by an improvement in both the general and local symptoms. If high fever exists, the temperature sinks. If collapse temperature is present, it usually rises. The subjective complaints, especially the sleeplessness, improve. The blood in the stools disappears; the movements of the bowels become less frequent and the severe pains concomitant with the same are absent. Finally, the consistency of the stools changes and at the end becomes normal.

Prophylactic use of the serum has met very favorable confirmation in the

work of Kruse, Vaillard and Dopter, and Rosculet. Rosculet's statistics are especially interesting. In 1905 during a dysentery epidemic in Roumania, Rosculet injected eighteen apparently healthy individuals living at the homes where dysentery cases existed, with 5 c.c. of the serum. Eighteen similar patients were removed from the dysentery surroundings, but received no serum. The results were that of the first group no fresh cases of infection arose, while of the control group fourteen were infected.

It is rather premature to determine definitely the value of the dysentery serum therapy; enough has been seen, however, to advocate its use whenever possible.

Staphylolysin.

Staphylolysin, or Staphylohemotoxin.—According to the experiments of M. Neisser and Wechsberg the pyogenes staphylococci produce a typical hemolysin which is identical for both the aureus and albus cultures. By immunization with this hemotoxin, an antihemotoxin (antilysin) is obtained. Neisser and Wechsberg further discovered that serum both human and of certain animal species normally contained antistaphylolysin, less, however, in amount than immune sera. Working on the principle that in staphylococcus diseases, a hemotoxin is formed which incites the development of antihemotoxin for the protection of the animal, Bruck, Michaelis and Schulze attempted to employ the presence of antistaphylolysin in the serum as evidence of the existence of Staphylococcus infections.

As staphylolysin, a twelve to thirteen day old bouillon culture of freshly isolated staphylococcus pyogenes serves very well. This can be preserved by adding 5 c.c. of the following mixture to 100 c.c. of the bouillon filtrate: 10 carbolic, 20 glycerin, 70 aqua. The hemotoxin content is approximated according to the following scheme:

Amount of filtrate.	Fresh rabbit blood.	Phys. NaCl.	Result of hemolysis after 2 hours in incubator at 37° and 24 hours in ice box.
0.2 c.c.	1 drop.	ad 2 c.c.	complete.
0.1 c.c.	1 drop.	ad 2 c.c.	complete.
0.05 c.c.	1 drop.	ad 2 c.c.	complete.
0.025 c.c.	1 drop.	ad 2 c.c.	complete.
0.01 c.c.	1 drop.	ad 2 c.c.	incomplete.
0.005 c.c.	1 drop.	ad 2 c.c.	layer of red blood cells.

Thus 0.025 is the smallest dose which can completely hemolyse the given quantity of red blood cells.

The amount of antilysin is estimated by adding varying amounts of serum to the constant minimal hemolytic dose of the staphylolysin and determining what amounts of serum contain enough antilysin to prevent

hemolytic action of the staphylolysin. It is best to allow the staphylolysin and serum to remain mixed for some time before adding the rabbit blood, so as to give the antitoxin a chance to neutralize the toxin.

As every normal serum contains a certain amount of antilysin, it is necessary in order to obtain the pathological variations, to use a normal serum as a control. Such a serum, 0.1 of which just suffices to neutralize twice the minimal hemolytic dose, was dried in vacuum and used by Bruck, Michaelis and Schulze, as standard serum.

Estimation of antilysin content of the standard serum:

Twice the minimum hemolytic toxic dose.	0.05	0.05	0.05	0.05	0.05
Standard normal serum.	0.2	0.1	0.05	0.025	0.01
Result after 24 hours....	No hemolysis.	No hemolysis.	Slight hemolysis.	Moderate hemolysis.	Complete hemolysis.

These mixtures were allowed to stand for one hour at 37° C. and then 1 drop of rabbit's blood was added, allowed to remain for two hours at 37° and twenty-four hours in the ice box.

The standard serum was always freshly prepared in the form of a 10 per cent. solution in distilled water (0.1:1).

In the above manner the antilysin content of the serum from patients with distinct or suspicious staphylococcus infections was estimated. The completely neutralizing dose of the standard serum (0.1 above) was taken as 1 and the neutralizing dose of the serum for examination compared with this; if 0.05 c.c. of a serum x neutralized the same amount of toxin as 0.1 of standard serum, the antilysin value of the serum x was 2.

From the comparative studies of Bruck, Michaelis and Schulze it was concluded that most of the normal sera had values ranging from 1 down; occasionally results as high as 5 were obtained. *Out of twenty-five cases of staphylococcus infections nineteen gave values varying from* 10 *to* 100. *Figures as high as these can, according these authorities, become of valuable interest and aid in diagnosis.*

Although these findings were corroborated by Arndt and others, this method cannot as yet be classed among those of clinical diagnostic importance. Similar study of other infections has not been undertaken.

In addition to the toxins reviewed in these chapters, recent work has proven that toxins may, under certain conditions be derived from bacteria other than those mentioned, *e.g.*, cholera, typhoid bacteria and meningococci. Problems such as these are still, however, open to scientific discussion; consequently no exact statements can be made here.

CHAPTER IX.

The Toxins of the Higher Plants and Animals and Their Antibodies. Ferments and Antiferments.

The toxins thus far studied were all secretory products of bacteria. This power of forming toxins is not, however, limited to bacteria alone, as there is a class of higher plants and animals that produce characteristic poisons against which immunization can be undertaken and an antitoxic serum obtained. Pollen toxin and snake poison are the only members of the groups which bear any practical medical interest. The detailed study of these plant toxins (Phytotoxin) and those of animal origin (Zootoxin) has, however, greatly increased the theoretical knowledge of the phenomena of reaction and immunity.

Phytotoxins.

The most important phytotoxins are:

1. Ricin.
2. Abrin.
3. Crotin.
4. Pollen.

Ricin. Ricin is a deadly poison, of which the smallest fractions of a milligram, are sufficient to kill rabbits. Like bacterial toxins, ricin requires for its action an incubation period of at least twenty-four hours. The typical post-mortem findings consist of redness and swelling of Peyer's patches. Ricin is a hemotoxin; if mixed, as an emulsion, with red blood-cells, the erythrocytes sink to the bottom and are agglutinated.

Ehrlich succeeded in immunizing animals against ricin by first giving it to them per os in increasing doses for a long period of time, and later on by subcutaneous injection. The antitoxic serum thus produced neutralizes the poisonous action of ricin both in vivo and in vitro.

Abrin. Abrin, a vegetable poison, is obtained from jaquirity (Abrus precatorius) and in its action closely resembles ricin, but is less poisonous. It is a marked irritant of the conjunctiva and was at one time employed in cases of trachoma.

Roemer found that by repeated instillation of abrin into the same conjunctival sac, no reaction was ultimately obtained (local immunity), while the conjunctiva of the other eye retained its susceptibility. If the instillation was continued for a long period of time, a "general immunity" was attained which extended to the conjunctivæ of both eyes. As a result, in the serum of such animals anti-abrin could be demonstrated.

Crotin.

It is the seed of croton tiglium that gives rise to crotin, a substance less poisonous than either ricin or abrin. It does not agglutinate, but hemolyses rabbits' red blood cells. Toward the red blood-cells of other species of animals (*e.g.*, bird), it is entirely inactive. The immunization of rabbits is readily brought about by subcutaneous injections. Their serum neutralizes the hemotoxic action in vitro.

Hay-fever.

The pollen toxin has been described by Dunbar as the etiological factor of hay-fever. In Germany the disease seems to come chiefly from pollen of the grasses and grains. (Rye pollen being most active); whereas in America, apparently, the most important pollen springs from members of the ambrosia (rag weed) and solidago (golden rod).

The toxin is isolated by mixing for ten hours the ground pollen with 5 per cent. NaCl solution and 0.5 per cent. phenol at 37° C. Then, in the form of a proteid it is precipitated by the addition of eight to ten volumes of 96 per cent. alcohol and the resultant white precipitate dissolved in physiological salt solution.

Susceptibility to the pollen toxin is limited only to certain individuals. Some are influenced by the rye pollen only, others by the golden rod alone, while a third class is affected by all. The cause for this peculiar idiosyncracy is unknown.

All those who suffer from hay-fever develop a marked conjunctivitis whenever even the slightest amount of pollen proteid (1/1000 mg.) is dropped into the conjunctival sac. In addition, all the symptoms of hay-fever or asthma may be incited. Similar effects are in evidence when subcutaneous injections are resorted to.

For purposes of immunization horses are most suitable, but only those which after an injection of pollen extracts manifest a local and general reaction. This is found in one-third of the cases. Their serum rendered immune is capable of neutralizing all effects of the pollen toxin.

As regards the standardization of this serum, it is effected by mixing the dosis minima certe efficax of the toxin with various dilutions of the serum and instilling the mixture into the conjunctival sac of individuals with a tendency for hay-fever. That amount of the serum which suffices to neutralize the toxic ravages is taken as the unit of measure. Sera of at least thirty times the unit strength are employed.

The immune serum is manufactured in fluid and powder form by Schimmel & Co. of Miltitz, near Leipzig, and is placed on the market under the name of "Pollantin." Its use is mainly local, and that by spraying a small quantity of the pollen powder upon the nasal mucosa several times daily and by placing several granules into the conjunctival sac with a camel's-hair brush. The serum can also be employed as a prophylactic.

If the eyes are especially reddened, it is best to deposit some fluid serum into the conjunctival sacs every day. Prausnitz advises the injection of 1

to 2 c.c. of the serum subcutaneously when asthmatic attacks occur, or when the above local treatment has failed.

In America a special Pollantin is made against the frequent form of hay-fever known as "autumn catarrh" by immunization with the pollen of the golden rod and rag weed.

The pollantin therapy and prophylaxis has been quite satisfactory, inasmuch as two-thirds of the patients remain either entirely free from attacks or are so greatly benefited that their general duties are not interfered with. The only radical means of curing the disease is a change of climate, suitable to the patient.

The Zootoxins.

Most important of the animal toxins are

1. Phrynolysin (toad poison), 2. Arachnolysin (spider poison),	} Simple hemotoxins.
3. Snake poison, 4. Scorpion poison, 5. Bee poison,	} Lecithin producing hemotoxins.

The one striking characteristic of toxins, that an immunity can be raised against them, is also possessed by these poisons. Beyond this fact they present many variations from the true class of toxins. Most of these poisons are complex, *i.e.*, they contain more than one toxin, and all are hemotoxic.

Toad poison is produced by rubbing up the skins of the Bombinator igneus; spider poison by trituration of the living "cross spiders" (Epeira diadema) in three or four times the amount of physiological salt solution containing toluol.

The toad and spider poisons contain simple hemotoxins, that is to say, by the mixture of small amounts of this toxin with erythrocytes absolutely serum-free, hemolysis of the latter takes place. Not all species of blood are affected alike. The red blood corpuscles of sheep, goats, and rabbits are especially adapted for experiments with phrynolysin, while rabbits', rats', and human blood is more suitable for arachnolysin. Immunity of rabbits is easily attained.

Snake-poisons.

The most familiar poisonous snakes are the Cobras (Naja) of India and Indo-China which belong to the family of Colubridæ, the European viper, and the American rattlesnake; the last two being of the Viperidæ species. The poisons of these two families show great individual differences. Thus, those of the Colubridæ group are decidedly thermo-resistant (temperatures

as high as 100° C.) while the viper's poison is entirely destroyed at a temperature varying between 80 to 85° C., and markedly weakened at 70°.

Snake poisons, as a rule, produce both local reactions at the point of the bite, and severe general disturbances.

The cobra bite is only slightly painful. A characteristic feeling of stiffness extends from the point of infection over the entire body. In several hours a rapidly increasing weakness sets in terminating in deep coma and death.

The viper bite incites a very strong local reaction. The point of infection is red, extremely painful and swollen. Convulsions and hemorrhages, followed by delirium which finally changes into stupor are manifest, and death takes place in one to three days. If the poison gets into the circulation directly, death is likely to occur in a few minutes.

The prognosis of a snake infection depends largely upon the situation of the bite. The greater the blood supply of the infected area the more dangerous is the result. Bites received through the clothing are relatively less dangerous, as a great part of the poison remains adherent to the clothing.

Snake poisons act primarily upon the nervous system and blood, although they exhibit a number of other toxic and ferment properties. Thus viper toxin occasions immediate coagulation of the blood by its action upon the vascular endothelium and has for this reason been called by Flexner and Noguchi, "Hemorrhagin."

Furthermore all snake poisons have a hemolytic power.

Cobra Hemolysis.

Cobra hemolysis represents one of the most interesting of biological phenomena, and as it may possibly be employed in clinical methods of examination its action will be here reviewed.

Cobra hemotoxin is characterized by its power of dissolving the red blood corpuscles of certain kinds of animals (ox, sheep and goat) only in the presence of serum. Other red blood cells do not require any serum for their hemolysis (dog, guinea-pig, man, rabbit, horse). If the red blood corpuscles of the first group of animals washed free of their serum are mixed with cobra poison, no hemolysis takes place. On subsequent addition of any fresh serum, hemolysis is in evidence. (Flexner, Noguchi).

The agent which instigates the hemolytic substance belongs undoubtedly to the class of lipoids. Of these, lecithin stands pre-eminent. It is, however, by no means certain whether that is the only or the most important activator.

Some sera exhibit this activating influence only when first heated. In their unheated state they are entirely inactive. Other sera act in a manner decidedly the reverse. Kyes and Sachs mention that this depends altogether upon the nature of the lecithin union in respect to the other elements present. The following table shows the various combinations and their resultant action.

Combination.		Power of serum activation.			
Serum.	Red cells.	Unheated.	Heated at		
			56°	65 to 100°	
Horse	Ox	+	+	+	
Horse	Horse	+	+	+	
Ox	Horse	+	−	+	
Ox	Ox	−	+	+	
Sheep	Ox	−	+	+	
Sheep	Sheep	−	−	+	weak hemolysis.
Human	Ox	−	−	+	
Human	Human	+	+	+	
Rabbit	Ox	+	+	+	
Guinea-pig	Ox	+	−	+	
Guinea-pig	Rabbit	+	−	+	

+ Signifies hemolysis.
− Signifies no hemolysis.

Cobra Hemolysin Test.

1. *Washing of Erythrocytes.*—The blood is collected into sterile flasks containing sterile glass beads. It is then shaken and thus defibrinated to prevent coagulation. The defibrinated blood is next centrifugalized and the serum separated and drawn off by means of a pipette. The red blood cell sediment is then mixed with physiological salt solution and again centrifugalized. This procedure is repeated several times until all the serum is removed. The red blood-cells as used are in a 5 per cent. suspension; *i.e.*, 1 part of washed erythrocytes suspended in 19 parts of saline.

2. *The Activating Agent.*—In order to obtain an activating agent 0.2 of serum or a 0.1 per cent. of a lecithin solution is employed. The lecithin can be kept as a stock solution consisting of 1 g. lecithin in 100 c.c. of methyl alcohol. A 0.1 per cent. solution of the stock mixture is made by mixing 0.1 c.c. of the solution with 9.9 c.c. of physiological salt solution.

3. The snake poison, hemotoxin, is resistant toward heat so that it may be heated to almost 70° C. without interfering with its activity. Cobra poison contains the greatest amount of hemotoxin. While 1 mg. of cobra toxin hemolyses 1 c.c. of 5 per cent. horse's red blood-cells in five to ten minutes, a similar amount of viper toxin requires thirty minutes for the same action.

V. Dungera and Coca explain this type of hemolysis on the ground of the existence of a ferment within the snake poison which breaks up the lecithin with the liberation of oleic acid. This acid has long been known as a hemolytic agent. The necessity for adding lecithin or serum to certain species of blood is explained by the variability in the lecithin content of the erythrocytes.

Cobra Toxin Activation in Tuberculosis.

Calmette notes in the blood of tuberculous patients more than the normal amount of lecithin; for that reason their serum can be used in very small doses to activate the cobra hemolysin. By this means he has found it possible to attain a diagnostic reaction for tuberculosis. On examination of the blood of 177 tubercular individuals he has found:

78 per cent. of positive reactions in the first stage of tuberculosis.
57 per cent. of positive reactions in the second stage of tuberculosis.
70 per cent. of positive reactions in the third stage of tuberculosis.

Szaboky has confirmed these findings, but not enough control examinations of normal individuals or of other infections have been made to firmly establish the diagnostic value of the test.

The hemolysis of snake poison can be overcome or interfered with by the addition of large amounts of normal serum, cholesterin, and small amounts of snake poison serum.

The Psycho-reaction.

Much and Holzmann have recently described the so-called "Psycho-reaction" which can be explained thus—Normal serum, when added to a mixture of cobra extract and human red blood-cells will not interfere with consequent hemolysis. If, however, the serum obtained is from patients suffering from depressive mania, circular insanity or dementia pracox, and added to the cobra extract and human red blood-corpuscles, the expected hemolysis does not take place. One would naturally suppose that this fact would be employed for clinical diagnosis, but unfortunately it has been generally proven by most authorities in this line that it is altogether impossible to do so for the simple reason that it is not absolutely specific. Bauer has found the same reaction with navel blood. It is probable that the interference with hemolysis is brought about by an increase in the cholesterin of the serum—a possibility in diseases of the central nervous system more so than under any other physiological or pathological conditions.

Cobra Immunity.

In immunizing laboratory animals one cannot start, at the beginning at least, with inoculations of the unaltered snake poison.

Phisalix and Bertrand begin by employing subcutaneous injections of a toxin heated to 75° C. and after two days use one-half of the minimal lethal dose of the unaltered toxin.

Calmette weakens the cobra poison by the addition of an equal amount of 1 per cent. gold chlorid, and after four such injections with increasing amounts at each time, the pure toxin in very small doses is employed.

In the same manner Calmette immunized horses and obtained highly antitoxic sera. He tested the strength of these sera, as follows:

1. *Upon Rabbits.*—Each animal received an injection of 2 c.c. of the serum into the vein of one ear, and after two hours 1 mg. of toxin into the vein of the other ear. A control animal was similarly treated with toxin only. The latter animal died in a half hour, while the former remained alive.

2. *Upon White Mice.*—Diminishing amounts of serum were mixed in test tubes, with 0.0001 gm. of toxin (in 1 per cent. solution) and the mixtures injected into the mice. The greatest amount of serum which completely neutralizes the toxin must be 0.03 c.c.

According to Calmette one can approximate the efficiency of an immune serum by its antihemolytic power inasmuch as the hemotoxic and neurotoxic actions run parallel. This is denied by Noguchi.

The scorpion and bee poisons display properties similar to those of the cobra poison. They also combine with lecithin to produce hemolysis.

Thus far, it has been shown that the lipoids, especially lecithin are actively associated in the hemolysis of erythrocytes; whether the toxin combines with the lipoids and forms a toxolipoid (toxolecithid) which is hemotoxic, or whether as v. Dengen believes, the hemolytic action is due to the fatty acid derived from the lecithin by the ferment action of substances contained in the poison, has not been definitely proven.

Quite recently it has been thought that pernicious anemia and paroxysmal hemoglobinuria are closely associated with such toxolipoids.

Pernicious Anemia.

Tallquist obtained from a Bothriocephalus latus, a hemotoxic poison of a lipoid nature which experimentally produced a blood picture characteristic of pernicious anemia. But it would be incorrect to associate all forms of pernicious anemia with tape worm poison; more probable is it that hemotoxins are formed within the organism itself.

Paroxysmal Hemoglobinuria.

In *paroxysmal hemoglobinuria* a hemotoxin of very peculiar properties is found circulating in the blood.

It can be demonstrated as follows.

1. *Ehrlich's method.*—One of the patient's fingers is ligatured by means of a small tourniquet and kept immersed in ice-cold water for a half hour. Some blood is then collected into a capillary pipette from the finger thus tied, and as a control, blood from a finger of the other hand is drawn off. This is allowed to clot and then centrifugalized. The results are that the serum from the finger held in the ice water is tinged red from dissolved hemoglobin while the control serum is normally pale.

2. *Donath-Landsteiner's* method repeats Ehrlich's experiment in vitro. The patient's and the control individual's serum are each mixed with washed human erythrocytes in various proportions. It does not matter whether the red blood cells are obtained from the patient or normal individual. The mixtures are allowed to remain for one-half to one hour in the ice box and then from one to three hours at a temperature of 37° C. The serum from the paroxysmal hemoglobinuria patient shows hemolysis.

A control tube containing the same ingredients, in the same proportions and maintained at *either* cold *or* warm temperatures, but not at both in succession as above, exhibits no hemolysis.

The hemolytic process in this disease is of a complex nature. In the cold, one element combines with the erythrocytes, and at high temperature another unfolds hemolytic tendencies. Some sera lacking or not having enough of the second element in the serum, demonstrate no hemolysis.

But on addition of some normal serum hemolysis occurs. It can therefore be concluded that the second factor which acts in the heat is present within normal serum, while the first substance, the specific one, is found only in the blood of those suffering from paroxysmal hemoglobinuria; (and according to Donath and Landsteiner in 10 per cent. of cases of general paralysis). It is, in addition, the author's opinion, that similar toxic substances exist in the blood of epileptics and idiots.

Not all cases of paroxysmal hemoglobinuria possess this characteristic hemotoxin. In some it is only found periodically.

No explanation has as yet been offered for these varying phenomena. Attempts have been made to ascertain whether the hemotoxin is stimulated by an external agent or by infection (Lues, malaria, trypanosomiasis) or whether it is of endogenous origin. The answer is still for the future to disclose.

The Antiferments.

Ferments are very closely allied to toxins in their biological structure. By the immunization of animals with ferments in as pure a form as possible, antiferments can be demonstrated. Just like antitoxins, antiferments can neutralize their respective ferments in vitro. As to their presence, it is quite important to know that they are found in normal serum in certain small quantities (together with antitoxins). The difference in their presence in a normal serum and that in an immune, is purely a quantitative one.

The antiferments thus far demonstrated are

Antilabferment.	Antipepsin.
Antitrypsin.	Antisteapsin.
Antifibrinferment.	

It is difficult to obtain by immunization an antiferment serum of very high strength. Probably the normal organism is so regulated that it compensates any increased amount of antiferment.

Antitrypsin.

Till recent times the demonstration of antiferments bore no clinical interest. The antibodies of the proteolytic enzymes first began to attract attention when the inhibitory influence, which blood serum has upon the autolysis of organs was proven. It was Jochmann and Müller who showed in connection with their studies of the proteolytic ferments of leucocytes, that apart from these, the serum itself possesses an inhibitory influence upon the leucocyte ferment. This is found to be especially marked in diseases associated with great destruction of leucocytes. Following them, Marcus, as well as Brieger and Trebing discovered a restraining influence in the serum upon the action of pancreas trypsin and proved that the so-called antitrypsin was considerably increased in carcinoma patients. Bergmann and Meyer, working also along these lines, then demonstrated that the wrongly called "carcinoma reaction" was by no means specific for carcinoma, but was found in a large number of other diseases. It cannot, as Brieger later announced, even be considered as a criterion for cachexia (cachexia reaction).

Brieger's Cachexia Reaction.

Undoubtedly, the already normal antiproteolytic power of the serum

can be considerably increased in animal experimentation by a group of well-known proteolytic agents, and especially by leucocyte ferment and pancreatic trypsin. To differentiate between antileucocyte and antitrypsin ferment in the narrow sense of the word, is impossible. The one "immune serum" (sit venia verbo, if one can speak of immune serum in this sense) neutralizes the other antigen. Clinically a high antitryptic titer of the serum is found in about 90 per cent. of carcinoma patients, and is almost regularly observed in infections with high fevers as typhoid, severe articular rheumatism, sepsis, etc. In pneumonia there is found during the infection a marked change from an excessively high to a low titer. In Morbus Basedow (as well as in experimental thyroid feeding) it is almost the rule to find a high antitrypsin content, but one must always keep in mind that even few normal individuals show a similar increase.

The clinical diagnostic import of the antitrypsin titer is slight in comparison with its experimental increase. In accord with the findings in Basedow, and in thyroid feeding it may be considered as an outcome of increased proteid destruction (hyper-production of proteolytic ferments in the tissues?) Leucocyte ferment has been found of practical use in the treatment of cold abscesses, *i.e.*, in processes where leucocytosis and failure to produce polyneuclear leucocyte ferment is present. On the other hand antitrypsin or antileucocyte ferment or even normal serum is employed to counteract inflammatory processes, *i.e.*, to neutralize the excessive production of the leucocyte ferments, with apparent success (Leucoantifermentin, on the market). According to recent findings, the antitrypsin titer of the mother's blood increases markedly during the period of labor, while that of the fetus remains unaltered.

There are two methods for the antitrypsin determination. The first was devised by Jochmann and Müller for proving the presence of leucocyte ferment and its antiferment, and then similarly employed by Marcus in the study of pancreatic ferments. Its principle depends upon the digestive action of proteolytic ferments upon serum albumin. When a drop of trypsin is placed upon a Löffler's serum plate, after a little while, a clear spot appears where the trypsin was brought into contact with the plate. If to this trypsin, an amount of serum is previously added, which fully neutralizes the digestive action, no clear zone appears upon Löffler's plate.

The details of this procedure are as follows: The ferment solution consists of 0.1 gm. trypsin, well shaken with 5 c.c. of undiluted glycerin and 5 c.c. of distilled water, then left in an incubator for a half hour at 55° C., then again shaken and filtered.

The serum is mixed in small test tubes or upon a glass slide with varying amounts of the trypsin; thus 1 loopful of serum is mixed with 1/2, 1, 2, 3, 4, etc., up to 20 loopfuls of the trypsin solution and of each of these mixtures one loopful is placed upon Löffler's plate. (Ox serum plate should be three days old). The plates are then placed into the incubator for twenty-one hours at 55° C. The presence or absence of the clear zones determines the quantities of ferment which respectively have not or have been neutralized by the one drop of serum (*e.g.*, 1 : 6 means that in the mixture of 6 loopfuls of trypsin and 1 loopful of the serum for examination the digestive power of the trypsin was still interfered with).

The inequality in the strength of the Löffler plates, their variability in the degree of alkalinity, the measurement by loopfuls, all, might prove to be sources of error which may greatly influence the results. Thus the latter can only be taken as approximate, relative values.

The second, more exact and satisfactory method was introduced primarily by Gross and Fuld for presenting the action of trypsin, and was modified by v. Bergmann together with Bamberg and Meyer for the determination of antitrypsin. Numerous workers have found it thoroughly reliable. Its principle is based upon the digestion of a clear casein solution. If the entire amount of casein is digested, no more is left to be precipitated by the addition of acid and therefore the solution remains clear. If, however, casein has been left undigested, the addition of acid will produce a turbid solution or even a white precipitate.

The necessary reagents are:

1. *Casein Solution.*—One gm. of casein is dissolved under slight heating in 100 c.c. of N/10 Na O H; this solution is next neutralized by N/10 HCl, litmus being used as indicator, and diluted with physiological salt solution up to 500 c.c. (If sterilized, it can be kept for a long while).

2. *Trypsin Solution.*—0.5 gm. of trypsin (purissimum Grübler) is dissolved in 50 c.c of NaCL+0.05 c.c. of normal sodium hydrate solution and then diluted with physiological saline up to 500 c.c.

3. *Acid Solution.*—Five c.c. of acetic acid+45 c.c. of alcohol+50 c.c. of water.

First the titration of the trypsin solution is undertaken in order to find out how much trypsin is required to fully digest a constant quantity of casein. Gradually increasing amounts of trypsin (from 0.1 to 0.6 c.c.) are placed into six test-tubes and to each 2.0 c.c. of casein are added. These tubes are placed into an ncubator at 37° for one-half hour, and then several drops of the acid solution are placed into each tube. The first tube, and all those above it that remain absolutely clear, contain enough trypsin to fully digest the 2.0 c.c. of casein.

Now comes the second part of the test.

Into each of eight to ten test-tubes, are placed 2 c.c. of the casein solution and 0.5 c.c. of a 2 per cent. dilution of the serum for examination; to these is next added the trypsin solution in successively increasing amounts, beginning with the smallest quantity which in the first part of the test was sufficient to completely digest the given amount of casein. Salt solution is then added to each of the test tubes so that all contain an equal quantity of fluid, and the mixtures placed into an incubator at 37° for one-half hour. At the end of this time, several drops of the acid are added to each tube. Those tubes which become cloudy or show a precipitate, designate the amounts of trypsin solution which have been neutralized by the 0.5 c.c. of diluted serum. For example:

In the first part of the test it was found that the tube containing 0.4 c.c. of trypsin was the first to remain clear, in other words was sufficient to fully digest 2 c.c. of the casein solution. In the second part of the test the lower limit of the added trypsin dilution was 0.4, and it was found that the tubes containing 0.4, 0.5, 0.6 and 0.7 c.c. of trypsin, for example, now gave precipitates and only 0.8 remained clear. This indicates that part of the formerly sufficient amount of trypsin was now neutralized by the antitrypsin of the added serum so that digestion was interfered with. Thus the antitrypsin titer in this case is 0.8.

Recently the above method of trypsin titration has been applied to the determination of the presence of pancreatic ferment in the intestine, feces, and stomach contents.

CHAPTER X.

AGGLUTINATION.

The Phenomenon of Agglutination.

If the serum from an immunized animal or a patient convalescing after an infection, be mixed with a suspension of the bacteria which were involved in the production of said conditions, a peculiar phenomenon takes place. In the formerly diffusely cloudy liquid, small granules and clumps appear which sink to the bottom of the test-tube and leave a supernant clear liquid. On microscopic examination, the sediment presents bacteria (which have remained alive as is demonstrable by making cultures of same). This same observation can be made when the experiment is performed in a hanging drop with perhaps more flattering results. The bacteria are seen to lose their motility, adhere to each other, finally gravitate toward larger groups and arrange themselves in clumps. The phenomenon thus described was discovered by Gruber and Durham, and is called *agglutination;* while substances which cause this, *agglutinins.*

If instead of the immune or that of the convalescent patient, normal serum is employed, and the above test repeated, it will be seen that agglutination likewise occurs. The reaction here is, however, somewhat incomplete, the clumps smaller, and formed much more slowly. *If a quantitative determination with different dilutions* of both sera is made, the power of agglutination disappears with the normal serum at a low dilution, while the immune serum remains perfectly active at even much greater dilutions. Thus the main difference between the agglutinating normal and immune serum is a quantitative one depending upon the amount of agglutinins present. Whether any qualitative difference exists between the normal and immune agglutinins is doubtful. It is, however, of no practical significance.

If instead of homologous bacteria, different (heterologous) bacteria are employed, *e.g.*, cholera vibrio and typhoid serum, agglutination also takes place, if the typhoid serum is used in concentrated or only slightly diluted form; but in moderate or great dilutions, no agglutination occurs. Normal serum will agglutinate the cholera vibrio in the same strength as the immune typhoid serum. In other words the typhoid serum contains more agglutinins for its homologous bacteria, than a normal serum, but it has only the same titer of agglutination as a normal serum for heterologous bacteria.

The agglutination reaction is specific in the respect that weak dilutions of

serum will agglutinate only its homologous bacteria and leave the heterologous ones uninfluenced. Agglutination becomes non-specific, when concentrated or strong dilutions of serum are employed.

Diagnostic Value of Agglutination.

The relative specificity just described is of great clinical diagnostic value. For example, given a serum suspicious of typhoid the question is to establish this absolutely. One immediately proceeds to make a suitable dilution of the unknown serum and mixes with it known typhoid bacilli. A similar dilution of normal serum is also made as a control and mixed with the same amount of typhoid bacilli. If agglutination occurs with the unknown serum and not with the control serum, the former must have come from a typhoid patient. If the bacteria are not agglutinated, the serum was not of typhoid origin.

In an equal manner can the identity of unknown bacteria be established by the use of known sera. Thus, when certain bacteria have been isolated and information is wanted as to whether same are of a typhoid nature, an emulsion of these is made and mixed with a typhoid serum in suitable dilution, and a similar amount of bacteria is mixed with a normal serum of like dilution. Agglutination occurring in the first of these mixtures and absent in the second, proves the typhoid character of the unknown bacteria. In this manner the agglutination test can be used for identification of an antigen.

The practical application of agglutination has been greatly made use of in cases of typhoid fever. Here the production of agglutinins is very easily stimulated in the course of the disease and generally they can be demonstrated in the serum seven to ten days after infection. The agglutinins remain not only during the active stage of the disease, but also during the convalescing period. Widal, the Parisian clinician, was the first to adopt this agglutination reaction for the serum diagnosis of typhoid. It is thus commonly known as the Widal reaction.

Technique of Agglutination.

The technique of the reaction is as simple as its principle. This accounts for its wide adoption. It may either be performed macroscopically or microscopically (orientation test).

The Macroscopic Agglutination Reaction.

The necessary requirements for carrying out the reaction consist of:

1. The immune serum and a normal control serum;
2. A homogeneous bacterial emulsion.

The production of a homogeneous bacterial emulsion offers slight technical difficulties.

It can be obtained in the following ways:

a. *Bouillon Culture.*—Many bacteria, like typhoid, paratyphoid, dysen-

tery, coli, etc., grow very easily in broth. Such a fresh (twenty-four hours), diffusely turbid culture can be employed readily for agglutination purposes. In place of live bacteria, dead may also be used—a fact which has greatly added to the practical application of the test.

For obtaining the latter, 0.5 per cent. of phenol or 1 per cent. of formalin (40 per cent.) are added to the twenty-four hour bouillon cultures. The result is, that a sediment of bacteria is formed from which the supernatant fluid should be carefully poured off. The bacterial suspension is kept on ice and thoroughly shaken before use.

Ficker has in this way prepared standard emulsions of dead typhoid and paratyphoid bacilli which are sold by Merck under the name of "Ficker's Diagnosticum."

For carrying out Widal's test, a small quantity of the patient's blood is collected into a capillary tube and the end closed with sealing-wax. The blood is allowed to clot, and the serum to separate off. The separation of the latter can be hastened by centrifugalization.

In practice, the Widal test as performed with Ficker's diagnosticum, is arranged as follows:

Bacillus suspension.	Dilution of serum.	Physiological salt solution.
Tube 1, 0.5 c.c.	.....	0.5 c.c.
Tube 2, 0.5 c.c.	0.5 c.c. of 1 : 10	.....
Tube 3, 0.5 c.c.	0.5 c.c. of 1 : 50	.....
Tube 4, 0.5 c.c.	0.5 c.c. of 1 : 100	.....

One of four results may be obtained.

1.	2.	3.	4.
Positive reaction.	Doubtful reaction.	Negative reaction.	Worthless reaction.
1. No agglutination.....	No agglutination.....	No agglutination.....	Agglutination.
2. Marked agglutination....	Marked agglutination..	Very slight agglutination.	Agglutination.
3. Marked agglutination....	Very slight agglutination.	No agglutination.....	Agglutination.
4. Slight agglutination.....	No agglutination.....	No agglutination.....	Agglutination.

It is very advisable to make control tests also with normal serum. After the mixture of the various ingredients the tubes are placed into the incubator at 37° for two hours. Then the results are read off and the first tube must show absolutely no agglutination otherwise (as seen in Division No. 4 above), the entire test is of no significance. The cause for such spontaneous or pseudo-agglutination occurring in tube 1, may be found either in the bacterial emulsion or NaCl solution. The grade of agglutination is estimated by the size of the agglutinated clumps and the rapidity with which they are formed. The mild grades of agglutination are frequently overlooked by the beginner.

For typhoid a positive reaction is one where agglutination takes place in the dilution of 1 : 100; a positive result in the dilution of 1 : 50 can only be considered as probably positive.

As has been said, broth cultures may be used for the agglutination test if the bacteria grow diffusely and regularly within the bouillon. This is not the case, however, with all bacteria, as for example, the cholera vibrio which produces a thin pellicle upon the surface of the broth.

b. *Agar Cultures.*—Kolle and Pfeiffer have advised, instead of broth the use of agar cultures. The bacteria are washed off, and an even emulsion made in physiological salt solution, or in a dilution of the serum for examination.

The details of the procedure are as follows:

Into a row of test-tubes is placed 1 c.c. of various dilutions of the serum for examination, *e.g.*, 1 : 10, 1 : 50, 1 : 100, 1 : 200, 1 : 500. A normal serum is similarly diluted as a control. In this respect only the higher concentrations of the normal serum are necessary. One other test-tube is to contain 1 c.c. of saline only.

A full loop of an eighteen to twenty-four hours' old agar culture is evenly and finely rubbed up in each of the above test-tubes as follows:

The test-tube is held almost horizontally in the left hand between the thumb and index finger; a platinum loop between the thumb and index finger of the right hand is filled with the bacteria from the agar culture, and placed into the tube containing the serum dilutions. The bacteria are then gently and thoroughly rubbed up upon the moistened wall of the tube but not within the fluid. By rolling the test-tube slightly, a part of the rubbed up bacteria is washed into the fluid and the remaining bacterial mass is again triturated. This process is repeated until all the bacteria are washed into the fluid. Thus, a homogeneous suspension is obtained.

The author has found this method of Pfeiffer and Kolle most accurate.

It is worthy of note in this connection, that the controls show no clumps or granules. (Pseudo-agglutination). There are some bacteria which can be evenly emulsified only with great difficulty, while others are very easily agglutinated even by normal serum. In either case the test is not conclusive.

For the hanging drop method, blood is collected into a Wright capsule or a small test-tube; 6 to 8 drops of blood suffice. The blood is allowed to clot or the serum is hastened by centrifugalization. Four loopfuls of broth or saline (or equal amounts as measured by a Wright pipette) are placed upon each of two slides. To one of these one loopful of the serum (or one equal part as measured by the Wright pipette) is added and thoroughly mixed. From this mixture one loopful or equal measure is mixed with the broth or normal saline upon the second slide; thus making serum dilutions of 1:5 upon the first slide and 1:25 upon the second slide. A loopful of typhoid culture is placed upon the center of each of two cover slips. To the first is added one loopful of the serum dilution 1:25, and to the second is added one loopful of the serum dilution 1:5 thus making a dilution of 1:50 and 1:10 respectively. Each cover slip is inverted over a hollow slide protected

by vaseline, and examined microscopically. A control with normal serum and also one with culture alone should be made.

Production of Agglutinating Sera.

For the identification of bacteria only *highly agglutinating animal sera* can be employed. Rabbits, goats and horses, are most suitable for such experiments. The best results are obtained when the animals are immunized intravenously by repeated injections with gradually increasing doses of dead bacteria (killed at 60° C.). Usually two to three injections of 1/4 to 1 agar culture suffice to give an agglutinating titer of 1 to 5000. The serum should be withdrawn eight to ten days after the last injection. As a matter of course the titer of the serum should be tested from time to time, because the height of the titer curve can only reach a certain point. When a sufficient strength is obtained the animal is bled. It is not possible to produce equally strong agglutinating sera for all bacteria.

The agglutinins belong to the class of the more resistant serum substances. With slight addition of carbolic acid they can be preserved on ice for a long time. Heating, variously affects the different bacterial agglutinins. The agglutinins for pest and tubercle bacilli are destroyed at 56° C. while other bacteria are not influenced by even higher temperatures. The animal from which the agglutinating serum has been obtained also influences to a great degree, the resistance towards heat. Thus the typhoid agglutinating serum derived from the horse is much more resistant than that obtained from the rabbit.

The Microscopic (Orientation) Agglutination Test.

This method is especially of use, when only small amounts of culture or serum are obtainable. Also, if agglutination is employed for the quick recognition of bacteria, as for example, when it is desirable to know whether a blue colony on a Conradi-Drigalski-agar plate is typhoid or not.

In such a case a drop of the immune serum in the dilution of 1:50 or 1:100 is placed upon a cover-glass held with a Cornet's forceps, and a small part of the bacterial colony for identification carefully mixed with this serum. As controls, a mixture is made with salt solution and with normal serum. If agglutination occurs, small granules or clumps can readily be seen with the naked eye by holding up the cover-glass against the light. The control glasses on the other hand should show only a homogeneous turbidity. These changes are still more evident if the mixture is examined microscopically in the form of a hanging drop. (Described, p. 100.)

Group Agglutination.

On testing the titer of a strongly agglutinating typhoid serum, and a strongly agglutinating cholera serum, against typhoid, paratyphoid, colon and cholera bacteria, the results will appear to be the following:

Agglutination titer.	Of typhoid serum.	Of cholera serum.
Against typhoid	1 : 2000	1 : 10
Against paratyphoid	1 : 100	1 : 10
Against bacter. coli	1 : 25	1 : 10
Against cholera	1 : 10	1 : 3000

The cholera serum acts strictly in accordance with the rules stated above for specific agglutinins, *i.e.*, marked agglutination with homologous bacteria; very weak, with heterologous. The typhoid serum on the other hand, although in the main it fulfills the same requirements, nevertheless manifests some important differences when mixed with heterologous bacteria. It has practically no influence upon the cholera vibrio with which the typhoid bacillus is not at all related; its agglutination of 1 : 10 can be attained even by a normal serum. The colon bacillus which closely resembles the typhoid, morphologically, but which has very different biochemical properties is more strongly agglutinated, 1 : 25; while the paratyphoid bacillus, very much like the typhoid bacillus, both morphologically and biologically is agglutinated even in larger dilutions, 1 : 100. This entire phenomenon, is an expression of the biological relationship of the various bacterial groups and is known as *group reactions*.

Partial Agglutination.

An understanding of group reactions is to be found in a more complete conception of specificity. From this source we have learned that the difference in antibodies is influenced by the dissimilarity of the injected antigen. For example, the difference between the cholera and typhoid agglutination is caused by that existing in the protoplasmic structure of the respective bacteria. As these bacteria, however, are not constituted of a distinct chemically defined substance, but made up of a mixture of various substances there may be a number amongst them which can act as antigens. If, figuratively speaking, there are five different elements in the body of the typhoid bacillus which can act as agglutinogens, *i.e.*, antigens, these should be able to form according to the law of specificity five different agglutinins. On mixing a typhoid serum with typhoid bacilli, one brings together five distinct antigen antibody combinations and consequently complete and thorough action results of this union. A biological relationship of bacteria implies the existence of some common protoplasmic constituents. Expressed in the same figurative manner the colon bacillus can be said to have antigen number 1 in common with the typhoid and paratyphoid bacillus and the paratyphoid may have antigen numbers 1 and 2 in common with the typhoid bacillus. As a result, *the typhoid* serum will react with colon bacilli by virtue of their common

agglutinin number 1, and with paratyphoid bacilli through its agglutinins numbers 1 and 2. The other "partial agglutinins" remain inactive on account of the missing suitable agglutinogens.

The existence of such partial antigens and partial antibodies is, for some bacteria more than of mere theoretical importance. It is even possible that a strong colon serum will agglutinate no colon bacilli other than that particular strain employed for the production of the serum. Such being the case with a number of micro-organisms, the sera made at present both for diagnostic and therapeutic purposes are polyvalent (multipartial). By polyvalent serum is meant one which is produced either by immunizing animals with many different strains of the same bacterium, or a mixture of sera obtained from different animals immunized with various strains.

Castellani's Test.

The practical importance of partial agglutinins is recognized in the diagnosis of mixed infections. Castellani found that by the mixture of an immune serum with its corresponding bacteria, the agglutinins for these as well as the partial agglutinins for the heterologous bacteria are absorbed. On the other hand, if the same serum be mixed with the heterologous bacteria, the agglutinins for the homologous group are quantitatively retained.

A practical example will make this clearer.

The serum of a patient agglutinates typhoid as well as paratyphoid bacilli, in a dilution of 1 : 100. This may indicate one of three possibilities:

a. Patient is infected with typhoid, but has formed an exceptionally large number of partial-agglutinins for paratyphoid bacilli.

b. Patient is infected with paratyphoid bacilli, but has formed at the same time many partial-agglutinins for typhoid.

c. Patient has a mixed infection of typhoid and paratyphoid and therefore formed agglutinins for both.

A decision in regard to the above may be reached according to the following method given by Castellani:

Four rows of test tubes are arranged, each row containing three tubes with 1 c.c. of serum dilutions 1 : 10, 1 : 50, 1 : 100 respectively. In each of the first and second row, 1 loopful of typhoid bacteria is emulsified.

In each of the third and fourth row, 1 loopful of paratyphoid B bacilli is emulsified.

The tubes are placed into the incubator for two hours, absence or presence of agglutination in each test-tube noted, and after centrifugalization (which may become unnecessary if the bacteria are strongly clumped or grouped at the bottom of the tube), the supernatant liquid is transferred into other test-tubes and kept in the same order.

Then each of the first row receives 1 loopful of typhoid bacilli,
each of the second row receives 1 loopful of paratyphoid B. bacilli,
each of the third row receives 1 loopful of typhoid bacilli,
each of the fourth row receives 1 loopful of paratyphoid B. bacilli,

All are once more placed into the incubator for two hours.

a. If typhoid exists, the agglutination titer in the second part of the test will become weaker in the first, second and fourth rows, while that in the third row remains the same.

b. If paratyphoid exists, the titer for typhoid in the first and third row becomes less,

that for paratyphoid in the fourth row diminishes, while the titer in the second row for paratyphoid remains the same.

c. If a mixed infection exists, the agglutination titer in the first and fourth row diminishes and in the second and third row remains the same.

In this connection a few exceptions may be mentioned:

Agglutinoids.

A serum which is kept for a long time, frequently loses part or even all of its agglutinating titer. Whereas it formerly agglutinated in the strength of 1:1000, it may now become inactive in dilutions even of 1:10. The first thought that arises in explanation of this is that the serum has perhaps degenerated and the agglutinins destroyed. If, however, further dilutions are made, 1;100 may show mild, while 1:500 strong agglutination. This, first of all, demonstrates that agglutinins are still present, although diminished in amount, and second, that another substance has arisen which in the stronger concentrations interferes with agglutination. A simple experiment explains this.

If the test-tube containing the serum dilution 1 : 10 and the non-agglutinated bacteria be centrifugalized, the serum removed and the bacteria mixed with a known strongly agglutinating serum, it will be found, that the bacteria have become inagglutinable. Substances of certain kinds have combined with the bacteria and prevented them from undergoing agglutination. These substances are strongly specific, acting only upon homologous bacteria. Their origin can also be demonstrated.

An agglutinating serum which is heated to 65° or 70° C. loses its agglutinating power but the substance interfering with the subsequent agglutination has remained. Ehrlich explains the situation as follows: He claims that agglutinins are built complexly; that they possess a binding (haptophore) group by means of which they unite with the bacteria (agglutinogen) and a second group (ergophore or agglutinophore) by virtue of which agglutination results. If serum is kept for a long period of time, or exposed to high temperature, many of the ergophore groups are rendered inactive, while the haptophore groups being more resistant remain with their full potentialities, and unite with bacteria. Agglutinins possessing only their haptophore groups are known as agglutinoids. They combine with the bacteria, and still do not agglutinate them, but at the same time prevent other agglutinins from acting. If this old agglutinoid and agglutinin containing serum is diluted, so few of both of these substances remain that the bacteria can absorb both, allowing the relatively few agglutinins to manifest their activity.

It is important to note in this respect, that occasionally even a fresh, highly valent serum, will present a tendency towards interfering with the agglutination processes. This is also explained by the existence of agglutinoids—a fact as yet not definitely proven.

Another finding, only encountered in exceptional cases, is the existence of the so-called *non-agglutinable strains* of bacteria. These give all the characteristics of the general class of bacteria to which they belong, but are not agglutinated by their respective serum; as, for example, a strain of typhoid bacilli, which are not agglutinated by any typhoid serum. The only positive proof that they are typhoid bacilli, is the ability to produce by their employment an active immunity against fully virulent typhoid bacteria.

Non-agglutinable strains of bacteria can be isolated especially from the lower animals. At times, however, they regain their agglutination property when they are grown in

artificial media and frequently subplanted. Possibly, the reason that the bacteria become inagglutinable at all, is that they undergo immunization within the organism against the existing agglutinins. By growing bacteria in agglutinating serum for a certain time, one can obtain inagglutinable strains.

Agglutination in Typhoid and Paratyphoid.

1. Agglutinins for *typhoid* and *paratyphoid* A and B, can, not infrequently, be demonstrated in the patient's serum as early as the third day, but as a rule, at about the beginning of the second week of the disease. Moreover, they remain within the serum for several weeks after the illness and disappear only gradually. A positive agglutination test does not, however, mean the existence of the corresponding disease. *A healthy bacillus carrier can also have an agglutinating serum.* Some cases of icterus catarrhalis even give a positive Widal test. But in order to assign to this last a correct explanation, one must remember that typhoid bacilli may remain in the gall-bladder for years and thus lead to catarrhal inflammation and stone formation.

Partial agglutinins from coli infections must always be considered. Some authorities mention a positive Widal, in connection with endocarditis maligna, sepsis, malaria, phthisis, and miliary tuberculosis.

An absence of the agglutination test, especially at an early part of the illness, should not influence a negative diagnosis of typhoid too greatly, inasmuch as many cases are known where the reaction appeared for the first time during the period of convalescence. In the employment of this test as an aid for the differential diagnosis between several bacterial infections, it is best to titrate the serum to its limit, as the higher titer for one class of bacteria generally speaks in favor of the infection by the same. Paratyphoid serum agglutinates typhoid bacilli only slightly, while true typhoid, both typhoid and paratyphoid bacteria with equally high force. In severe and difficult cases, Castellani's test should be performed. Paratyphoid B. serum, always gives the limit of its agglutinating titer both with the pathogenic mouse typhoid and hog cholera bacillus.

2. *Cholera.*—Only rarely has the agglutination test been employed with the serum of patients thus afflicted. On the other hand, the identification of cholera suspicious colonies in the stool is regularly conducted by means of this test. For this purpose, it is very specific, as group reactions almost never take place. Strongly agglutinating sera are easily obtained by immunization of animals.

3. *Epidemic, Cerebrospinal Meningitis.*—Agglutination in this disease serves mainly for the identification of suspicious meningococcus cultures. As has been shown by Wassermann and Kutscher, some strains are agglutinated only after a long period (twenty-four hours) and at higher temperatures as 56° C.*

*Frequently, during even the first days of the disease, the patient's serium in a dilution of 1–10 gives the agglutination test. This is rare with higher dilutions of the serum as 1–50. It usually takes some time before the agglutination becomes evident.

4. *Dysentery.*—The agglutination property is employed both for testing the serum, and identifying cultures. The Flexner type of bacillus, produces agglutinins more readily than that of Shiga-Kruse. They are also agglutinated more readily. Only positive reactions in dilutions of 1:30, are of diagnostic consideration. Occasionally partial agglutination takes place with heterologous dysentery strains, typhoid and colon bacteria.

5. *Pest.*—The reaction is very specific, but of slight significance, as it appears only upon the ninth day; occurring with a serum dilution of 1 : 3, it is considered of positive diagnostic value.

6. *Malta Fever.*—In most instances the serum gives the agglutination reaction with the micrococcus melitensis. Normal serum may give the reaction in dilution 1 : 30, so that higher dilutions only are of aid in diagnosis.

7. *Staphylo, Strepto and Pneumococci.*—Clinically, the agglutination test is never employed in these cases.

8. *Tuberculosis.*—Here the agglutination test is associated with the difficulty of obtaining a homogeneous tubercle bacillus suspension. This, however, is overcome by one of two ways.

a. Arloing-Courmont's Method (1898).—The tubercle bacilli are obtained in the so-called "*homogeneous culture*" form. S. Arloing first grows the bacteria upon potatoes for a long time, and then transplants them into glycerin bouillon which is agitated, daily for five minutes. After a number of subcultivations, a culture is finally obtained after several months. This grows rapidly in a few days and diffusely clouds the broth.

Such a culture diluted with physiological saline solution, is used for the test. Here small test-tubes are preferable and the ingredients should be mixed after the following proportions:

2 drops of serum + 10 drops of culture (1 : 5)
1 drop of serum + 10 drops of culture (1 : 10)
1 drop of serum + 15 drops of culture (1 : 15)
etc.

The combined substances are well shaken and placed into an incubator. According to Arloing and Courmont, a positive reaction even in the dilution of 1 : 5 speaks for tuberculosis. Best results are by this means obtained in incipient and mild tubercular cases; those which are farther advanced do not react.

b. Method of Koch.—Koch filters the ordinary tubercle bacillus bouillon cultures, dries the remnants upon the filter, and rubs them up in an agate mortar with N/50 NaOH up to a dilution of 1 : 100. The solution is centrifugalized and enough weak HCl is added until the reaction is only slightly alkaline. The dilution is then brought up to 1 : 3000 by the addition of 0.5 per cent. phenol in normal saline, and kept for twenty-four hours in the incubator.

A somewhat simpler procedure is to dilute new tuberculin B. E. to 1 : 100 with 0.5 per cent. of carbolic saline solution, centrifugalize this for six minutes and then dilute to 1 : 1000. The solution thus obtained can be preserved in the ice-box for fourteen days. Just before using, a still further dilution of 1 : 10 is made.

The agglutination test has not been generally adopted as a method for diagnosis. The technique is rather difficult, and the results not absolutely reliable. The reason for the latter is that high agglutination values are rarely met with, and slight ones are found even in normal individuals. Then, too, the methods of tuberculin diagnosis are so much simpler, that they have been given the preference.

Koch himself advised the agglutination test, not as a means of diagnosis, but rather as an aid in tuberculin therapy. He found that during the treatment of tuberculosis with new tuberculin the agglutinative power of the patient's serum increased. He therefore took this as an index of the acquired immunity. Further study, however, convinced him that the agglutination cannot thus be interpreted, so that at the present day tuberculosis agglutination has no practical application.

10. *Glanders.*—Highly valent sera can be obtained, according to Kleine, by intravenous immunization of donkeys and goats. The serum serves for identification of the glanders bacilli. Kleine prepares a standard bacterial emulsion in the following manner: Four well grown glanders cultures are killed at 60° C. and the mass of bacteria triturated in 2 c.c. of 1/2 per cent. carbolic-saline solution. This is then diluted in a measuring glass so that 40 to 50 c.c. of carbolic-saline solution are added for each culture. The entire mixture is filtered through paper and 3 c.c. are used in each test-tube. Normally, horses may have an agglutination titer up to 1 : 400. Glanders infected animals react as high as 1 : 2000. Injections of mallein increase the agglutination titer. Experiences in this respect with the human being are still scanty.

Hemagglutinin.

Just as injections of bacteria produce bacterial agglutinins, injections of erythrocytes stimulate the formation of hemagglutinins which cause the red blood cells to congregate in clumps.

At times the presence of hemagglutinins is masked by the simultaneous existence of hemolysins which dissolve the red blood corpuscles. If, however, the immune serum is heated to 56° C. the hemolysin is destroyed, thus allowing the agglutinins to exhibit their action. In other instances as during the immunization of rabbits with dog's erythrocytes, hemagglutinins are formed in such great quantities that in mixing the immune rabbit's serum with the dog's erythrocytes so strong an agglutination occurs that the hemolysins can no longer attack the clumped erythrocytes. The hemolysin presence can be demonstrated only if clumping is prevented mechanically, by thorough shaking of the mixture.

CHAPTER XI.

Precipitins.

In the former chapter, the phenomenon of agglutination was explained as a clumping of bacteria occurring when a serum is mixed with its corresponding bacteria. In 1897 R. Kraus described a phenomenon, very closely allied to the one just mentioned. He found that when an immune serum, for example, of cholera, typhoid, or pest, is mixed with the clear, sterile filtrate of the respective bouillon cultures of their bacteria (instead of the bacteria themselves), the clear solution becomes turbid, and a precipitate forms. This reaction is known as precipitation, the elements within the immune serum, precipitins; while the substances (antigen) with which the precipitin reacts and which originally stimulated the production of the precipitin, *precipitinogen*.

Like all biological reactions, the phenomenon of precipitation is not limited to bacterial immune sera and culture filtrates, but is observed when any animal, vegetable or bacterial soluble proteid substance, is mixed with the serum of an animal which has been immunized against the particular proteid material in question.

Tschistowitsch and Bordet were the first who called attention to these non-bacterial precipitins. Bordet (1899) found that the blood serum of rabbits treated with the serum of chickens gave a specific precipitate when mixed with chicken serum. Tschistowitsch demonstrated a similar reaction with the sera of rabbits treated with horse's and eel serum.

The biological structure of the precipitins is strongly analogous to that of agglutinins. Many authorities, in fact, consider them identical. Whatever has been said in regard to the effects of heating and addition of acids or alkalies upon agglutinins, applies equally to precipitins. Moreover, they also are composed of two groups, a binding (haptophore) and a functionally active (ergophore) group. If the latter is missing, they are known as precipitinoids, and can interfere with precipitation just as agglutinoids do with agglutination.

In speaking of precipitation, it has always been customary to differentiate between bacterial and proteid. For practical purposes this division is superfluous inasmuch as the bacterial precipitins are nothing more than precipitins of bacterial proteids.

Bacterial Precipitin Reactions.

Production of Precipitin Sera.

For the production of precipitating sera, animals are immunized either with the bacterial bodies themselves, or fluids containing the bacterial proteid (precipitinogen), such as the filtrates of bouillon cultures and the various forms of bacterial extracts. The serum from individuals undergoing an infection or convalescing from one, contains precipitins against the respective infective agent.

Inasmuch as the reaction consists of the formation of a precipitate, it is important that *both of the ingredients (precipitin and precipitinogen) be absolutely clear and have no tendency to spontaneously become turbid, or form a precipitate.*

Obtaining Clear Sera.

In order to get a clear serum one should avoid withdrawing the blood during the period of digestion of the animal, because it is chylous at such a time. In man the best occasion for obtaining the blood is in the morning before breakfast. As for animals, it is advisable to give them no solid food (or milk) for twenty-four hours previous to venesection. Then a very minute quantity of blood is withdrawn and immediately centrifugalized in order to ascertain whether the serum is clear or not. If it is satisfactory, larger amounts may be collected. Erythrocytes and bacteria produce, at some times, turbid serum. Simple sedimentation or centrifugalization suffices to overcome this.

If in spite of these precautions turbidity still persists, recourse may be had in filtration through paper or bacterial filters, preferably new ones. This method should, however, be used as a last resort, because filtration always tends to diminish the strength of a serum.

Bacterial Precipitinogens.

Bacterial precipitinogens are prepared by filtration either of bouillon cultures or bacterial extracts. The filtrates must be absolutely clear; also sterile, as frequently the reaction requires a long period of time. If bacteria are present they may grow quickly, and produce turbidity. The precipitinogen loses after a time its property of combining with precipitins and forming precipitates. In such a case the precipitinogen can be employed for immunization purposes.

Technique of the Reaction.

A constant amount of precipitinogen is placed into each of a row of test tubes, and to these are added diminishing amounts of the serum.

A set quantity of serum and varying amounts of precipitinogen can also be employed. The result of the reaction depends to a very large extent upon the quantitative relationship of these ingredients. *If relatively too much precipitinogen exists, a precipitate will not form.* An already formed precipitate will dissolve on the addition of more precipitinogen.

The explanation of this peculiarity is unknown. Since colloidal substances, however, at times give similar reactions, many authorities have classed the precipitins among them.

The carrying out of a precipitation test is best seen in the following table:

Cholera bouillon filtrate.	Cholera serum.	Physiological saline sol.	Result.	
			After 4 hours.	After 24 hours.
5.0 c.c.	1.0 c.c.	—	Very cloudy.	Clear; marked sediment at bottom.
5.0 c.c.	0.5 c.c.	0.5 c.c.	Cloudy.	Clear with moderate sediment at bottom.
5.0 c.c.	0.1 c.c.	0.9 c.c.	Faint cloud.	Clear; slight sediment.
5.0 c.c.	0.05 c.c.	0.95 c.c.	Clear.	Clear; no sediment.
5.0 c.c.	—	1.00 c.c.	Clear.	Clear; no sediment.
—	1.0 c.c.	5.0 c.c.	Clear.	Clear; no sediment.
—	0.5 c.c.	5.5 c.c.	Clear.	Clear; no sediment.
—	0.1 c.c.	5.9 c.c.	Clear.	Clear; no sediment.
—	0.05 c.c.	5.95 c.c.	Clear.	Clear; no sediment.

A parallel row of tubes with normal serum should be included.

If highly valent sera, such as are obtained by immunization with bacterial extracts, are employed, precipitation may result soon after mixing the two constituents. The precipitins are strongly specific, although it may be said that just as in agglutination, *there exists in precipitation a certain degree of "group reactions."*

Diagnostic Value of Bacterial Precipitation.

The precipitation test has no clinical diagnostic value. It demonstrates nothing more than the agglutination test, is more difficult of execution and associated with greater sources of error. Only occasionally is it of service to prove the presence of soluble bacterial substances within exudates or organ fluids.

Porges and v. Eisler have employed the precipitation test as a means for the differentiation of capsule-bacteria where the method of agglutination is associated with certain difficulties. The precipitinogen was produced by filtration of four-weeks-old bouillon cultures of pneumococci, rhinoscleroma, and ozoena bacilli. The immune serum was obtained from rabbits which had received four to five subcutaneous injections of the bacterial suspensions.

Fornet has recently advocated the precipitation test as an aid in the clinical diagnosis of typhoid. Although his attempts have not been attended with practical success, the principles of the reaction deserve discussion on account of their originality.

Fornet believed that it should be possible to demonstrate in the blood of typhoid patients the presence of the antigens (precipitinogens) which stimulate the antibodies, long before the latter themselves become evident. He actually was able to obtain turbid

mixtures when he combined precipitating typhoid serum with the serum of typhoid patients. In many cases he obtained these results before the appearance of the Gruber-Widal reaction.

The method which he has recently employed is known as the "ring test."

Fornet's Ring Test.

Small test-tubes 8 cm. in height and 0.5 cm. wide, are placed in rows of twenty each into a small black test-tube rack so arranged by the help of side stands that the tubes are inclined at an angle of about 45°. Across the back of the rack is attached a strip of dark cloth as a background to facilitate the detection of any precipitate. The immune (or convalescent) serum is placed into different tubes in concentrated and diluted form 1 : 5 and 1 : 10 with normal saline, and then the serum for examination in concentrated and similar dilutions is carefully floated on top of the immune serum. The mixtures are allowed to stand undisturbed at room temperature for two hours, and if the reaction is positive a whitish ring at the point of contact of the two sera, makes its appearance. A control test-tube of normal serum plus immune, and another of normal plus the unknown serum in the same dilutions as those employed in the test, must remain negative.

Besides in typhoid, the ring test is also evident in scarlet fever, measles and syphilis.

Syphilis Precipitation.

In syphilis precipitation, the serum from patients with manifest luetic symptoms is employed as precipitinogen, and the serum from individuals with general paresis acts as precipitating agent. The ring test must be carried out strictly in accordance with the rules given by Fornet, but even so, its diagnostic value for syphilis is still doubtful. Plaut claims that normal serum gives the reaction just as often as luetic serum; this is strongly denied by Fornet.

Theoretically, it is questionable whether these precipitates and rings are similar in origin to bacterial precipitates, or whether physicial-chemical causes are at the bottom of the former phenomenon. In accordance with the latter view several other reactions have been recently recommended for the serum diagnosis of syphilis.

Porges' Reaction.

a. Porges and Meier noticed that luetic sera are capable of producing flocculent precipitates from lecithin solutions. Porges soon found the same occurrence with solutions of bile salts.

Many additions and modifications have been instituted in the case of Porges' reaction since it was first recommended. According to the most recent publication, the reaction is carried out as follows:

The requirements are:

1. One per cent. solution of sodium glycocholate (Merck) in distilled water.

2. The patient's serum which must be absolutely clear, and heated for one-half an hour at 56° C.

Two-tenths of each of the above are placed into a narrow test-tube 6 to 7 mm. in diameter, and allowed to rest for sixteen to twenty hours at room temperature. A positive reaction consists of the appearance of distinct coarse flocculi which as a rule, collect near the surface. Mere turbidity or faint precipitates are considered as negative.

The original Porges method of employment of lecithin was not at all specific, the reaction being present in tuberculosis, carcinoma, and other infectious diseases. As for the new modifications, nothing has been brought forward in their support or non-support.

Klausner's Reaction.

This reaction belongs to the same general class of precipitation tests for lues, but is very much simpler than any of the others. Two-tenths c.c. of absolutely clear, fresh (at the most, two hours old), active serum is mixed with 0.6 c.c. of distilled water, in a small test-tube 7 × 0.5 cm. Sera containing hemoglobin or lipoids are not suitable for this reaction. The mixtures are allowed to stand at room temperature. In several hours, at the latest fifteen, a thick flocculent precipitate 2 to 4 mm. high appears at the bottom of the tube. Kreibich's analysis showed it to consist of fibrin globulin.

Apparently this substance is increased in luetic serum and precipitated by the distilled water in which it is insoluble. Klausner's reaction is by no means specific for syphilis as it is in evidence in starvation, typhoid, measles, scarlet, pneumonia, and other diseases, as well as during health. Nevertheless it must be said that it is found more frequently, earlier and much stronger in lues than in any other condition.

Klausner states that in fresh cases of lues the best reaction is seen in about seven to nine hours, while in older cases a week reaction appears in twelve hours. Mercury influences the test in that the interval until the precipitate becomes marked, is prolonged and later on the precipitate becomes fainter.

In spite of its simplicity, Klausner's reaction has not been generally adopted for clinical work, inasmuch as the far greater accuracy of the Wassermann reaction has made the latter invaluable.

Proteid Precipitins.

While bacterial precipitation is interesting from a biological standpoint but bears no practical significance, proteid precipitation represents one of the most important practical aids in forensic medicine. *By this means the differentiation of various proteids can be easily and definitely determined, a problem which was left unsolved by chemistry.*

The phenomenon of protein precipitation is absolutely analogous to that of bacterial precipitation. If a clear proteid solution (a) is mixed with the clear serum (a') of an animal immunized against the above proteid (a), turbidity and precipitation will occur; while if a mixture of the serum (a') is made with a non-homologous proteid say (b), or a mixture of the proteid (a) with the serum (b') of an animal immunized against b, no precipitation takes place. Graphically expressed it looks thus:—

$a + a' =$ precipitation.
$b + a' =$ no precipitation.
$a + b' =$ no precipitation.
$b + b' =$ precipitation.

In other words, *a precipitating immune serum reacts only with its homologous proteid. The precipitin reaction is specific.*

Forensic Use of Albumin Differentiation.

It is greatly to the credit of Wassermann and his co-workers A. Schütze and Uhlenhuth, who recognized that this specificity of precipitins was of great medico-legal value.

Thus in a case where for example, a bloody shirt is found in the home of a man charged with murder, and the prosecution sees in that the proof of crime, while the defendant pleads that the stains belong to the blood of a sheep, the proof as to their source is of the utmost deciding evidence; and while chemical or microscopical examinations are here of little or no use, serum diagnosis wins the day.

The blood-stained clothing is extracted in water, part of the extract is mixed with *a*, the serum of a rabbit immunized against human serum and another part is mixed with *b*, the serum of a rabbit immunized against sheep's serum. If the mixture *a* shows a precipitate, it can be definitely stated that the blood stain contained serum derived from a human being; while if mixture *a* is clear and *b* shows the precipitate, it is strongly corroborative of the presence of sheep's serum.

Blood Relationship.

This example suffices to indicate the value of this biological fact. In addition the reaction is made use of in the determination of the nature of meats (detection of horse meat substitution for beef).

Furthermore, this method has explained a number of scientifically interesting problems. Just as group agglutination demonstrated the close relationship existant between various bacteria, so also serum precipitation proves a distinct relationship between the different species of animals (horse and donkey, dog and fox, hare and rabbit, ape and man, etc.).

Thus the serum of a rabbit immunized against human serum, precipitates not only human serum but also that of monkeys; the serum of a chicken immunized against rabbit's serum precipitates not only that, but also hare's serum. In order, however, to differentiate between rabbit's and hare's serum, Uhlenhuth advises the immunization of a rabbit with hare's serum. The serum of such an immunized rabbit, precipitates only hare's serum and not rabbit's, for the reason that "Isoprecipitins," *i.e.*, precipitins against the same kind of animal, are, as a general rule not developed. Similarly the differentiation between human and ape's serum can be accomplished by the immunization of ape's with human serum.

Attempts to determine the origin of albumin in urine, and the foreign proteids circulating in the blood of artificially fed infants have also been made by means of the precipitation reaction.

The technique remains the same, independent of the purpose it is employed for. It consists in the mixing of the clear precipitating serum and the clear proteid, or albumin precipitinogen.

In order to obtain accurate results, strongly precipitating sera must be had. These are best made by immunizing rabbits with the precipitinogen fluid (albumin solution, milk, meat juice, etc.). Three to four intravenous injections with 1 c.c. of the solution at intervals of six days usually suffice to produce a precipitating serum of high titer. The injections can also be given by the intramuscular or subcutaneous method, but here larger quantities are necessary.

It is advisable to inject five or six animals at the same time, instead of only one, inasmuch as rabbits vary greatly in their individual power to produce precipitins and moreover, because some die after the third injection. Frequently only one serviceable serum is obtained, even though the immunization of five rabbits was undertaken.

Beginning on the sixth day after the injection, one should, at regular intervals of one or two days, remove a small quantity of blood from the vein of an ear and test the strength of the serum. As soon as it is found to be satisfactory the animal should be bled and its serum preserved on ice, with precautions for sterility. The rules given above for obtaining a clear serum should be kept in mind.

If the serum is not withdrawn at the proper time, its strength begins to diminish and further injections no longer stimulate new antibodies. It is even possible for the entire precipitin action of the serum to disappear.

Titration. The following method of titration is the simplest. One c.c. of various dilutions (1:10, 1:100, 1:1000, 1:10000) of the proteid under examination (precipitinogen) is placed into different test-tubes and 0.1 c.c. of the precipitating serum is added to each. The tubes should not be shaken, but it is occasionally necessary to place them into the incubator for one hour before any turbidity or precipitate appears. The least amount of proteid solution which still distinctly shows a precipitate, is taken as the titer of the serum.

Uhlenhuth's Method of Proteid Differentiation. For medico-legal purposes, Uhlenhuth advises the use of only highly valent sera. He considers an antiserum as efficient if 0.1 c.c. of it, when mixed with its respective serum in the dilution of 1:1000, produces a distinct turbidity, either at once or one to two minutes at the latest; three to five minutes is the limit for an indication of turbidity in the dilutions of 1:10000 and 1:20000.

Like in all other biological reactions, control tests, here two in number, are of the utmost importance. One tube must contain 0.1 c.c. of the precipitating serum mixed with 1 c.c. of saline, another 0.1 c.c. of the precipitating serum mixed with a heterologous serum in the dilution of 1:200 and 1:1000. Both of these tubes should show absolutely no precipitate after twenty minutes. In this way the specificity of the precipitin is determined; and it must be remembered that it is the quantitative specificity which counts.

In the process of the determination of the nature of meats, it is especially necessary to ascertain exactly the precipitating titer against bovine and pig's serum possessed by the rabbit's precipitating serum directed against horse's serum.

When clear solutions are at hand the precipitin reaction is comparatively simple. Frequently, however, the test must be performed with old and dirty

blood stains, or all kinds of prepared sausage so that the first and important task is to obtain a clear solution.

In dealing with blood, milk, or seminal stains, the parts of the clothing involved are excised, divided into very minute shreds, and placed into a test-tube with a small amount of 0.85 per cent. of salt solution. If the material is not too old, extraction of the above nature, for one hour is usually sufficient, otherwise it may necessitate a period of twenty-four hours or more. Stains upon solid material such as steel, wood, stone, etc., are carefully scraped off, and suspended in physiological salt solution. To obtain a clear solution the extract must be passed through filter paper or eventually the lilliputian bacterial filter.

In the examination of meats, or other food stuffs, it is best to remove the material for examination from the center of its thickest part, as this portion has been least exposed to the methods of preservation, especially the high temperatures. Three hours extraction is usually sufficient; the fresher the meat, the shorter this period. Very much salted meats are best washed with distilled water, previous to extraction. Inasmuch as a great deal of fat interferes with the reaction it is advisable to remove it beforehand by extraction wth ether and chloroform for twenty-four hours (Miessner and Herbst).

Before performing the actual test with the unknown blood stain, it is best to try out the entire reaction with a similar but known blood stain in order to make sure whether all the ingredients are in good working order. In laboratories equipped for medico-legal examinations, stains made upon linens from the blood of man, ox, pig, horse, etc., are always kept in readiness for such preliminary tests.

Uhlenhuth indicates a set of rules to be observed whenever the reaction is undertaken. They are here cited in their original form, as practice has shown them to be of great service.

"In order to obtain sufficient extract for the test, a small amount of the material is placed into a test-tube containing 5 c.c. of normal salt solution. This must not be shaken. After one to two hours, 2 c.c. are poured off into another tube and gently shaken. If a persisting froth appears upon the surface of the fluid, it can be taken as proof that sufficient extraction has occurred, and the rest of the fluid is thereupon also transferred to this tube. If no froth appears the 2 c.c. should be returned into the first test-tube and the extraction continued until repeated tests finally show the presence of froth. It is preferable not to disturb the sediment at the bottom of the test-tube. The extract eventually obtained may have to be filtered, if not absolutely clear.

Such an extract is as a rule, stronger than that required for the test, *i.e.*, 1 : 1000. If, however, one drop of a 25 per cent. nitric acid solution is added to 1 c.c. of a 1 : 1000 serum dilution and then heated, a faint opalescence appears. Enough saline should therefore be added to the final extract so that the nitric acid test corresponds to that given by a dilution of 1 : 1000.

The following mixtures are then made:

Test solution.	Precipitating serum from rabbit.	Normal rabbit's serum	Normal saline.	Result	
				After five minutes.	After twenty minutes.
1 c.c. 1:1000	0.1	—	—	Opalescence.	Turbidity and sediment.
1 c.c. 1:1000	—	0.1	—	Clear.	Clear.
—	0.1	—	1 c.c.	Clear.	Clear.

The result should be read after twenty minutes at room temperature. As a further control a similar row of tubes should be made with the extract of the non-bloody part of the clothing in order to show that the latter alone does not give the reaction.

Even putrid or otherwise chemically changed proteids may still give the precipitin reaction.

The precipitation test only determines the animal species from which the proteid originates, but cannot prove whether it comes from the blood, semen, milk or other albumin body. In order therefore to make a medico-legal diagnosis of "human blood stains," chemical evidences must in addition be brought forward, that the stain really consists of blood. Obermeyer and Pick have further shown that besides animal specificity ("origin specificity"), precipitation also demonstrates the "constitutional specificity" of proteids.

"Origin" and "Constitutional" Specificity.

If instead of employing pure animal, or plant proteids for the immunization of animals, variously changed albumins are used (heated albumins, acid albumins, formaldehyde albumin, etc.) the organism reacts by producing antibodies of a characteristic nature, different from those developed after inoculation with the pure albumin. For example; the serum of a rabbit immunized for a long time with horse's serum (normal immune precipitin) will produce a precipitate when mixed in vitro with the pure horse's serum and not when added to the latter, heated, even if the normal immune serum is of very high titer. On the other hand, if a rabbit is injected with horse's serum which has been changed by being diluted and boiled for a short time, the immune serum thus obtained will react not only with native horse's serum but also with heated serum and a group of its decomposition products with which the normal immune serum ordinarily never induces a precipitate.

This fact is of practical application. In meat substitution, it is very popular to boil the sausage in order to make detection of the substituted meats more difficult. With the aid, however, of precipitins produced by immunization with heated proteids, this fabrication is more easily detected than if a normal immune serum were used.

Precipitin.

While animal specificity is not destroyed when the albumins are modified in the above manner or changed by tryptic digestion or oxidation, Obermeyer and Pick have demonstrated that their specificity is lost when an iodin, nitro or diazo group is inserted into the proteid molecule. Immunization with such transformed proteid compounds, *e.g.*, xanthoprotein, can produce a precipitating serum which will react with every xanthoprotein even in homologous animals. These authors conclude that species specificity is probably dependent upon a certain aromatic group of the proteid molecule.

It is interesting to note that the proteid contained in the lens of the eye belongs to this class of modified proteids which possess constitutional, but no species specificity. A serum produced by immunization with lens substance, will react with the proteid derived from the lens of any animal but with no other animal proteid.

Origin of Precipitate.

In conclusion, the origin of the precipitate formed during the precipitation reaction is of interest. When a very strong precipitating serum is employed, the precipitinogen is so greatly diluted that it no longer gives any of the chemical reactions for proteids, but nevertheless yields a heavy precipitate when the precipitating serum is added. This surely cannot come from the small trace of proteid in the precipitinogen. Furthermore, if the immune serum is diluted, the formed precipitate becomes comparatively weaker and disappears entirely if dilution is increased. It is, therefore, generally considered that the precipitate originates from the immune serum.

CHAPTER XII.

Bacteriolysins and Hemolysins (Cytolysins).

If a guinea-pig is immunized with living or dead bacteria, for instance cholera or typhoid, and then to test its immunity is injected with a single fatal or many fatal doses of living bacilli, the animal remains alive; whereas a normal-control animal, not treated beforehand, succumbs to a similar inoculation. In order to determine the forces to which the immunized animal owes its protection, Pfeiffer undertook the following experiment: Two guinea-pigs, one immunized and another normal, were simultaneously injected intra-peritoneally with living cholera vibrios, and the peritoneal exudate was withdrawn from time to time and examined microscopically in hanging-drop preparations. (The method of withdrawing the peritoneal fluid with capillary pipettes and other technical details will be described below.)

Pfeiffer's Phenomenon.

A very striking phenomenon occurred. While the cholera vibrios in the peritoneal exudate of the normal animal retained their form and motility and increased in number continuously until the animal succumbed to the infection, the bacteria in the peritoneal exudate of the immunized animal behaved quite differently; they first began to lose their power of locomotion, then their form changed, they broke up into evenly small shining masses, so-called "granula," and finally, after several minutes these also disappeared. Guinea-pigs injected with the peritoneal exudate from these infected immune animals remained healthy, and nutrient media inoculated with material from the same source remained sterile.

The above experiment is named after its discoverer, Pfeiffer, and the phenomenon itself, "bacteriolysis."

Bacteriolysis is a strictly specific process. If an animal which is immune to cholera is inoculated with typhoid bacilli, the bacteria markedly increase, as in a normal animal. The process by which this bacteriolytic force takes place is clearly demonstrated when a mixture of living cholera vibrios and blood serum of a guinea-pig which has been actively immunized against cholera, is injected into the peritoneal cavity of a normal guinea-pig and as a control, normal serum mixed with living cholera vibrios is inoculated into a second guinea-pig. Here the exudates on examination from time to time show that in the peritoneal cavity of the animal injected with the immune

cholera serum, the same phenomena of bacteriolysis occur as described above, leading to the sterilization of the peritoneal cavity, and protection of the animal from illness. In the control animal, however, the normal serum has no influence upon the bacteria, so that they increase rapidly and kill the animal.

It is evident then, that the bacteriolytic power resides not only in the actively immunized animal, but that it may also be transmitted to other animals by means of the former's serum. Bacteriolysis, therefore, is not a property of the tissues of the actively immunized animal, but is to be traced to specific antibodies, "Bacteriolysins" which circulate in the blood serum and body fluids.

From the above experiment it must be assumed that the phenomenon of bacteriolysis like agglutination and precipitation, can be demonstrated also in vitro. The earlier investigations in this connection, however, were unsuccessful. Bordet was the first to obtain conclusive results and also to elucidate the cause of previous failures.

While agglutination in vitro and bacteriolysis in vivo were readily produced by mixing living bacteria with old immune serum, bacteriolysis in vitro did not occur under similar circumstances. But when *freshly drawn* blood serum or exudate of an immune animal was used, bacteriolysis took place in vitro also. (In fact, granule formation can be directly observed by the microscope). When the serum becomes old—and twenty-four hours is sufficient to cause the change, it loses its bacteriolytic powers. It seems at first glance as if bacteriolysins may be active outside the body also, but that here they lead only an ephemeral existence. This view, however, is not quite correct; for "inactive" serum, which has become "ineffective" in vitro, can again produce bacteriolysis, if it is utilized to passively immunize healthy animals. Something must exist in the organism, which supplements the inactive bacteriolysins and restores their activity. This "reactivating substance" is independent of the immunizing process, since it is to be found in normal animals also. Furthermore, inasmuch as not only cholera and typhoid immune sera, but also any other immune sera and not only guinea-pig's serum but even rabbit's, horse's, and human serum may in like manner be reactivated, it is evident that the reactivating agent lacks specificity. On account of this peculiar quality of supplementing the inactive bacteriolytic serum so that it can develop its real effectiveness, Ehrlich called the reactivating substance "*Complement.*" *Accordingly, the complement is a normal non-specific substance which is found in the body fluids (particularly abundant in the blood serum) of every organism; its existence is evidenced either by the activation or reactivation of bacteriolytic antibodies.*

Bordet demonstrated that the apparent ease with which the bacteriolysins lose their activity is to be traced not to these bodies, but to the complement. If a small amount of fresh normal serum is added to bacteriolytic serum

which has become inactive, reactivation occurs in vitro, that is to say, the bacteriolytic serum, regains its ability to dissolve bacteria. The bacteriolytic power of fresh immune serum, depends, therefore, upon the fact that it contains not only bacteriolysins but also complement; the failure of old immune serum to produce bacteriolysis is accounted for by the lack of complement, while its capacity for reactivation is explained by the still present bacteriolysins.

As the above described experiments indicate, *bacteriolysis is a complex process, which is produced by the interaction of two substances; one, the bacteriolysin, is formed through an immunizing process, and accordingly is a specific antibody of great stability, while the other, the complement, is a normal non-specific and very labile serum substance.*

The stability of the immune bacteriolysin is evident in its resistance to heat, whereas the complement is thermolabile. If freshly drawn immune serum is heated to 56° C. for one-half hour, the complement is, as a rule, rendered ineffective, while the bacteriolysin is not in any way injured; it retains its specificity, and the degree of its affinity to antigen remains unchanged. Bacteriolysins are effected by temperatures above 60° C. only.

Concerning the finer mechanism of bacteriolysis there are two opposing views, that of Bordet and of Ehrlich. Without considering too closely the remarkable researches of these two investigators, the synonyms for bacteriolytic antibodies usually found in the literature will be reviewed.

In attempting an explanation of bacteriolysis, Bordet has recourse to certain phenomena in staining technique. There are some substances which can be stained only when prepared in a definite way by means of another substance, a so-called mordant ("Beize") which itself is not a stain. According to Bordet, the specific substance produced by immunization represents a kind of mordant which "sensitizes" the bacteria to the action of the second normal non-specific substance; the *latter* is really the active agent in causing the dissolution of bacteria and is called by Bordet "alexin"—an older term used by Buchner—in contradistinction to "substance sensibilitrice."

Ehrlich, on the other hand, advocates a more chemical conception of the essential process of bacteriolysis. He believes that the substance formed by immunization which for the sake of brevity, is called the immune body, is characterized primarily by the fact that it has two binding groups. One of these has a chemical affinity for the bacterial cell and is, therefore, known as the "cytophile group," the other is characterized by its binding affinity for complement and is, therefore, known as the "complementophile" group. Also because of its two binding groups (receptors) the immune body itself is called amboceptor, that is, double receptor.

Thus, according to Ehrlich, bacteriolysis takes place in the following way: The cytophile group of the amboceptor, which is strictly specific for its antigen, attaches itself to the antigen, for instance the cholera vibrio; while the complementophile group binds the complement. The complement must be regarded as a sort of digesting (proteolytic) ferment. Although it is always present in normal serum, it is not effective, because bacteria have no affinity for it. Only through the medium of the amboceptor, (Zwischen-Körper, intermediary body), can complement bind itself to bacteria and dissolve them.

The specificity of the bacteriolytic process depends, therefore, on the specificity of the cytophile group, while the complementophile group possesses no or, strictly speaking, only slight specificity; it adapts itself to the complements of very many though not quite all kinds of animals.

Technique of Bacteriolytic Experiments.

To determine the occurrence of bacteriolysis there are two methods of procedure;

1. Pfeiffer's experiment.
2. The bactericidal plate method.

I. The Pfeiffer's Experiment.

The essentials of Pfeiffer's experiment have been described at the beginning of this chapter. Briefly, it consists in injecting intraperitoneally into a normal animal, bacteriolytic immune serum mixed with living bacteria. The resulting bacteriolysis is studied microscopically by withdrawing small amounts of peritoneal exudate from time to time. If this experiment is performed with various dilutions of immune serum, and if it be determined at what dilution bacteriolysis fails to occur, then the bacteriolytic titer is evident.

The details can best be understood by taking a practical example. It is desired to find the bacteriolytic titer of the serum of a patient recovering from typhoid fever by means of the Pfeiffer experiment.

To accomplish this task the following ingredients are needed:

1. A strain of bacillus typhosus of known virulence for guinea-pigs.
2. Patient's serum, sterile, and free from complement.
3. Guinea-pigs of 250 grams weight.

A preliminary experiment must be performed in order to determine the virulence of the typhoid strain.

TESTING THE VIRULENCE OF STRAIN.

Guinea-pig No. 1.	1./II. 09 One loopful of a typhoid agar culture suspended in 1 c.c of bouillon, injected intraperitoneally.....	2/II dead
Guinea-pig No. 2.	1./II. 09 One-half loopful of same.....	2/II dead
Guinea-pig No. 3.	1./II. 09 One-fifth loopful of same.....	2/II dead
Guinea-pig No. 4.	1./II. 09 One-eighth loopful of same.....	2/II sick 4/II dead
Guinea-pig No. 5.	1./II. 09 One-tenth loopful of same.....	2/II sick 3/II well

As far as the Pfeiffer experiment is concerned the virulence titer in this case is 1/5 of a loopful of an agar culture because this dose is fatal within twenty-four hours. In order, however, to make sure of excluding all individual variations, which can and occasionally do occur, it is advisable to use not the titer dose, but its fifth or tenth multiple, that is, in this case, one loopful.

Doses larger than one loopful should be avoided, so that if any particular strain of typhoid bacilli is not sufficiently virulent, necessitating the use of larger doses, the virulence must first of all be increased. This is done by passing the organism through animals such as guinea-pigs.

To Increase the Virulence.

The method is as follows: A very large dose of the culture, for example the surface of an entire agar tube, is injected intraperitoneally. Every animal succumbs to this enormous dose. The bacteria-laden exudate from the abdominal cavity, which, of course, must be removed under sterile precautions is then inoculated into a second guinea-pig and when it dies, into a third, and so on. As a rule, after passing through one or two animals the bacterial strain (which must be grown pure from the cadaver) becomes more virulent, as can be proven by titration. Very often the virulence is increased exclusively for the species of animal used and occasionally this is associated with a decrease in virulence for other species. After a series of passages through animals, the strain reaches its maximum strength beyond which it cannot be increased. The degree of virulence varies with the type of bacteria. Typhoid and cholera usually reach only a moderate virulence (1/10 to 1/20 loopful); the bacteria of the hog cholera group can acquire a distinctly higher virulence; for instance, B. paratyphosus, 1/100 to 1/1000 of a loopful, while the streptococcus and pneumococcus reach the highest figures, 1/10000 to 1/1000000 of a loopful.

For the Pfeiffer's experiment with cholera or typhoid, the most suitable strains are those of such a virulence that 1/5 to 1/10 of a loopful injected intraperitoneally kills in twenty-four hours.

Technique of Pfeiffer's Experiments.

The serum to be investigated is freed of its serum by heating in a water-bath for one-half hour at 56° C. Then a series of dilutions are made in bouillon (not in salt solution) for instance 1/10, 1/100, 1/1000, etc. A c.c. of each dilution is put into a test-tube (a sterile pipette should be used) and rubbed up with a standard loopful of an 18- to 24-hour agar culture of typhoid bacteria. Finally the contents of each test-tube are injected intraperitoneally into a guinea-pig of 250 grams weight.

Inasmuch as small amounts are apt to be lost when aspirating the fluid with the syringe as well as when pouring the bacterial emulsion into a watch glass, it is better to rub up two loops of the culture in 2 c.c. of bouillon instead of 1 loop in 1 c.c., and then withdraw only 1 c.c. for use in the experiment.

The following controls should be prepared:

1. Dilutions of the serum of a normal person (or animal of the same type) + typhoid culture.

2. Dilutions of immune serum + a heterologous culture.
3. (a) Bouillon + typhoid-culture.
   (b) Bouillon + heterologous culture.

The study of the bacteriolytic phenomena follows the inoculation. For this purpose capillary pipettes to withdraw the peritoneal exudate are prepared according to the directions of von Issaeff.

A thin glass tube is heated in a Bunsen flame almost to the melting point, then removed from the flame and immediately drawn out with a sudden jerk. Very fine capillary pipettes can be thus made.

The removal of the exudate is accomplished as follows: a small cut is made with scissors through the skin of the guinea-pig's abdomen; the capillary pipette, the large end of which is kept closed with the index finger, is forced into the abdominal cavity with a single push. The pressure of the finger is next relaxed and the tube slowly withdrawn. In order to avoid injuring the intestines, the precautions usually advised in intraperitoneal inoculations should be observed here. The author has found Friedberger's method of holding the animal very serviceable (see Fig. 5). The procedure is absolutely painless, moreover, the ordinarily sensitive guinea-pigs withstand the operation almost without uttering a sound.

It is best to withdraw the exudate immediately after the injection and then at intervals of five to ten, twenty, and thirty minutes, etc. Observations are made directly in hanging-drop preparations. Stained specimens are less reliable and instructive because, according to the investigations of Radziewsky, the findings are dependent upon the kind of coloring matter used. Bacteria which are in the process of dissolution soon lose the power of being stained by methylene blue, while they retain their affinity for carbol-fuchsin and aqueous solution of gentian violet. The production of granules occurs only incompletely in stained preparations.

The prognosis for the animal quoad vitam, is unfavorable, if bacteriolysis does not occur; good, if it does. Yet there are exceptions to the latter rule, a subject to which reference will be made later on. Now that the most important technical details of the Pfeiffer phenomenon have been considered, the protocol following will more clearly illustrate their procedure.

Titration of a bacteriolytic serum (after Pfeiffer).

Guinea-pig No. 1.	1–4–07	One loopful of typhoid culture.	+0.1 typhoid serum. in 1 c.c. of bouillon intraperitoneally.	Beginning of bacteriolysis after 10 minutes; after 30 minutes exudate was sterile.	Animal remained alive.
Guinea-pig No. 2.	1–4–07	One loopful of typhoid culture.	+0.01 typhoid serum. in 1 c.c. of bouillon intraperitoneally.	Beginning of bacteriolysis after 15 minutes; after 20 minutes only isolated, non-motile bacteria, many granules; after 40 minutes sterile.	Animal remained alive.
Guinea-pig No. 3.	1–4–07	One loopful of typhoid culture.	+0.001 typhoid serum. in 1 c.c. of bouillon intraperitoneally.	Beginning of bacteriolysis after 15 minutes; after 20 minutes numerous granules and also many non-motile bacteria; after an hour, no bacteria at all.	2/4 animal slightly ill. 3/4 animal recovered and remained alive.
Guinea-pig No. 4.	1–4–07	One loopful of typhoid culture.	+0.0001 typhoid serum. in 1 c.c. of bouillon intraperitoneally.	After 20 minutes granules and many motile bacteria; after 1 hour motile bacteria very numerous.	2/4 animal found dead.
Guinea-pig No. 5.	1–4–07	One-fifth loopful in 1 c.c. of bouillon intraperitoneally.		After 20 minutes granules and many motile bacteria; after 1 hour motile bacteria very numerous.	2/4 anima found dead.
Guinea-pig No. 6.	1–4–07	One loopful.	+0.1 normal serum. in 1 c.c. of bouillon.	After 15 minutes many granules, also isolated motile and non-motile bacteria; after 20 minutes motile bacteria; after 30 minutes motile bacteria very numeours.	2/4 animal found dead.
Guinea-pig No. 7.	1–4–07	One loopful of bacteria, paratyphosus. (Virulence 1/10 loopful.)	+0.1 typhoid serum. in 1 c.c. of bouillon.	Completely similar findings.	2/4 animal found dead.

The bacteriolytic titer of the tested serum in this case would lie between 1 mg. and 1/10 mg. and could be exactly determined by further tests which would take into consideration the intermediate doses.

On close study of the above experiment, it will be noted that even in those cases in which the animals died of the infection, bacteriolytic phenomena were not altogether absent. They occurred particularly in the beginning and were incomplete. This can be considered as evidence of the fact that even normal animals possess a certain supply of bacteriolysins which are, however, readily exhausted. This amount of normal bacteriolysin in serum varies greatly with the species of animal; thus the sera of man and rabbit contain very little normal bacteriolysins for cholera and typhoid, while horse's serum is well supplied with the same.

According to Kolle, a loopful of virulent cholera vibrios is destroyed in the peritoneal cavity of a guinea-pig, by

0.005 to 0.01 c.c. of normal horse's serum.
0.01 to 0.02 c.c. of normal ass serum.
0.02 to 0.03 c.c. of normal goat's serum.
0.1 to 0.3 c.c. of normal rabbit's serum.

The protective action of bacteriolytic sera differs very essentially from that of antitoxic sera. For the latter, the law of multiple proportions holds true; a stronger dose of toxin is neutralized by a proportionately larger amount of antitoxin; to bacteriolytic sera this rule does not apply. If the bacteria are increased beyond a certain quantity, their dissolution can indeed be accomplished by the addition of sufficient amounts of (bacteriolysin), but the animal dies nevertheless. Its peritoneal cavity examined during life or post-mortem may be absolutely sterile. Pfeiffer's explanation for this phenomenon is that the endotoxins within the bacteria are liberated by bacteriolysis and kill the animal. Fatal results from endotoxin follow in a similar manner when dead instead of living bacteria are injected.

Endotoxin.

Since endotoxins can continue their effective action in spite of the serum, it is evident that the usual bacteriolytic serum lacks the power to neutralize the poisons of the endotoxins. Many investigators have attempted to supply this deficiency. (This will be considered later).

While bacteriolysis may take place without any resulting protective action, on the other hand a serum may be curative in spite of the *absence* of bacteriolysis. This is well demonstrated in Metschnikoff's experiment.

A marked leucocytosis in the abdominal cavity of a guinea-pig is produced by the intraperitoneal injection twelve hours previously of 5 to 10 c.c. of aleuronat solution or sterile bouillon. Pfeiffer's experiment is then performed. As a rule, bacteriolysis occurs also here up to a certain point, particularly when cholera vibrios are used; most of the bacteria, however, retain their form and are taken up by the leucocytes.

Metschnikoff's Experiment.

Metschnikoff used this experiment to uphold his theory of the signifi-

cance of phagocytosis. Pfeiffer maintained that bacteriolysis was the most important protective weapon of the immune organism against bacterial invasion. According to Metschnikoff and his followers among whom Bail in particular must be mentioned, bacteriolysis in the abdominal cavity is only an exceptional phenomenon (test-tube experiment in vivo); its occurence is made possible by the circumstance that the abdominal cavity is as a rule almost free of wandering cells, and that the few which are present are so injured by the severity of the infection, that they disintegrate. If their number increases, bacteriolysis does not occur, or at least is only slight. Likewise, bacteriolysis is incomplete in the presence of cells, for instance in the blood, spleen, liver and subcutaneous tissue, etc.

A detailed consideration of this much mooted problem does not fall within the compass of this book. It is sufficient to have pointed out the great questions of fundamental significance which hinge upon the discussion of the Pfeiffer experiment, questions which concern the essential features of antibacterial immunity. It can be readily understood, therefore, why the phenomenon of bacteriolysis has been so much studied, although its practical significance is only limited.

The Practical Application of the Pfeiffer Experiment.

The Pfeiffer experiment can be used in the differentiation of bacteria as well as in the demonstration of bacteriolysins in serum. It serves as a control for the agglutination reaction. Pfeiffer and Kolle, Brieger and others, have used bacteriolysis as a method of estimating the immunity obtained by active protective immunization against cholera and typhoid in man. It must, however, be questioned whether it is admissible to draw conclusions as to the degree of active immunity from the height of the bacteriolytic titer of the serum, inasmuch as animals are found which possess no active immunity and still have sera of high bacteriolytic powers.

The most important practical use of the Pfeiffer experiment lies in the identification of suspected cholera cultures. In Germany, the Pfeiffer test made with the vibrios obtained in pure culture from the suspected patients, is required for the official diagnosis of the first cases of cholera.

The serum used for this purpose should be at least strong enough in amounts of 0.0002 c.c. to cause the disintegration of the bacteria in one hour, when a mixture of one loopful of an eighteen-hour agar culture of cholera with 1 c.c. of nutrient bouillon is injected into the peritoneal cavity of a guinea-pig.

For this experiment four guinea pigs of 250 grams weight are used.

Animal.	Culture.	Serum.	Method of injection.	Result in cholera cases.
No. 1.....	One loopful of an 18 hours' growth of culture suspected to be cholera in 1 c.c. of bouillon.	0.001 c.c. cholera serum = 5 times the titer dose.	Intraperitoneally.	After 20 minutes or at the latest 1 hour bacteriolysis occurs; animal remains alive.
No. 2	One loopful of an 18 hours' growth of culture suspected to be cholera in 1 c.c. of bouillon.	0.002 c.c. cholera serum = 10 times the titer dose.	Intraperitoneally.	After 20 minutes or at the latest 1 hour, bacteriolysis occurs; animal remains alive.
No. 3 (control.)	One loopful of an 18 hours' growth of culture suspected to be cholera in 1 c.c. of bouillon.	0.01 c.c. of normal serum = 50 times the titer dose of the immune serum.	Intraperitoneally.	Increase in number of bacteria; animal dies.
No. 4 (control of virulence of culture.)	One-fourth loopful of an 18 hours' growth suspected of being cholera in 1 c.c. of bouillon.		Intraperitoneally.	Increase in number of bacteria, animal dies.

In cases of subsiding cholera, the Pfeiffer experiment is performed with the serum of the patient in dilutions of 1 to 20, 1 to 100 and 1 to 500.

Bacteriolysis with typhoid organisms is less typical than with cholera. For diagnostic purposes the test is resorted to, only when the agglutination reactions are doubtful. When bacteriolysis also gives uncertain results, an animal is immunized with the typhoid suspected bacteria and its serum tested for its power of agglutinating or destroying definitely known typhoid bacteria and eventually the immunized animal may be injected with virulent typhoid bacilli.

Bacteriolysis is even more unsatisfactory with bacillus paratyphosus and the related hog cholera group of organisms.

While in typhoid the onset of bacteriolysis offers a favorable prognosis for the animal, guinea-pigs inoculated with bacteria of the paratyphoid hog-cholera group die in spite of complete bacteriolysis. Death always takes place late (from three to six days), while the control animals succumb in about twenty-four hours. Bacteriolysis has also been observed with the bacillus of dysentery and with the tubercle bacillus; but thus far, these phenomena have gained no clinical significance. Bacteriolysis does not occur in anthrax, pest and the various diseases due to cocci.

II. Bactericidal Plate-culture-method.

(Plattenverfahren) *after Neisser and Wechsberg.*

For the determination of the bactericidal titer of a serum, *Neisser and Wechsberg recommended the so-called bactericidal plate-culture method.* The principle of it is as follows: the serum to be tested is inactivated; different amounts of this inactivated serum are mixed with a definite constant quantity of bacteria, and a constant quantity of active normal serum is added as complement. This mixture is left in the thermostat sufficiently long to permit the occurrence of bacteriolysis. Now, to determine whether and to what degree death of bacteria resulted from the effect of the reactivated bacteriolysins (or of some bactericidal substance otherwise unknown), agar is added, the mixture plated, and the number of colonies counted.

Stern and Korte recommend this procedure for clinical purposes, as a substitute for the Pfeiffer test in the diagnosis of typhoid. They point out the sparing of animals as one of its advantages. On the other hand, this method consumes much more time and its results are less trustworthy. It has not found a place, therefore, in clinical practice.

The Technique of the Method.

The technique of Stern and Korte is the following: the serum of the patient, and that of a person not ill with typhoid as control, are inactivated for one-half hour at 56° C. and 1 c.c. of each in decreasing dilutions is poured into sterile test-tubes. To each is added 0.5 c.c. of a twenty-four hour typhoid bouillon culture diluted in bouillon to 1 : 5000 or 1 : 10000. For reactivation 0.5 c.c. of fresh normal rabbit's serum in a dilution of 1 to 12 in physiological saline is added and the whole thoroughly shaken. The tubes are then placed into the thermostat for three hours. The entire contents of each mixture is plated in agar, and after eighteen to twenty-four hours the plates are to be examined. That particular plate is considered to indicate the end value of the bacteriolytic action of the serum in which there is evident a very great decrease in the number of colonies as compared with the innumerable colonies found on the control plates.

Certain other controls are necessary:

1. One tube containing culture and complement.
2. One containing culture and inactivated immune serum in the highest concentration used.
3. The same with inactivated normal serum instead of immune serum.
4. Complement without culture and immune serum to test its sterility.
5. Immune serum without culture and complement to test its sterility.
6. One tube containing only culture, to be plated immediately.
7. One tube, containing only the culture, to be plated after standing in the thermostat for three hours.

Töpfer and Jaffé pour a thin layer of agar into a petri dish and let it harden. Upon this the culture-serum-agar mixture is poured, and after hardening is covered with another thin layer of agar. In this way the formation of a film of culture in the water of condensation is avoided.

A practical example is appended to illustrate the plate culture method.

Culture.	Serum.	Complement.	Result (poured after remaining 3 hours in the thermostat).	
			Normal serum.	Immune serum.
0.5 1/5000 typh.	1/100 c.c.	0.5 1:12 rabbit's.	0 colonies.	Many thousand.
0.5 1/5000 typh.	1/500 c.c.	0.5 1:12 rabbit's.	100 colonies.	Many thousand.
0.5 1/5000 typh.	1/1000 c.c.	0.5 1:12 rabbit's.	Many thousand.	Many thousand.
0.5 1/5000 typh.	1/5000 c.c.	0.5 1:12 rabbit's.	∝	Many thousand.
0.5 1/5000 typh.	1/10000 c.c.	0.5 1:12 rabbit's.	∝	500
0.5 1/5000 typh.	1/20000 c.c.	0.5 1:12 rabbit's.	∝	200
0.5 1/5000 typh.	1/30000 c.c.	0.5 1:12 rabbit's.	.....	0
0.5 1/5000 typh.	1/40000 c.c.	0.5 1:12 rabbit's.	.....	5
0.5 1/5000 typh.	1/50000 c.c.	0.5 1:12 rabbit's.	.....	60
0.5 1/5000 typh.	1/100000 c.c.	0.5 1:12 rabbit's.	.....	800
0.5 1/5000 typh.	1/200000 c.c.	0.5 1:12 rabbit's.	.....	∝
Control I 0.5 1/5000 typh.	—	0.5 1:12 rabbit's.	Many thousand.	
Control II and III 0.5 1/5000 typh.	1/100 c.c.	—	∝	∝
Control IV—	— .	0.5 1:12 rabbit's.	0	
Control V —	1/100 c.c.	—	0	
Control VI. 0.5 1/5000 typh. immediately poured.	—	—	Many thousand.	
Control VII 0.5 1/5000 typh. poured after 3 hours.	—	—	∝	

In addition to the results which one would expect, this experiment shows one striking point. With the normal serum the tube which contains the largest amount of normal bacteriolysins shows on plating, the fewest germs. The greater the dilution of the serum the more prolific is the bacterial growth. The titer of the normal serum in this case lies between 1/100 and 1/500. The controls show that the serum and complement are sterile, and that the inactive normal serum is ineffective. During the three hours in the thermostat the bacterial suspension has become stronger. The retarded growth in the complement culture tube can be traced probably to the presence of normal bacteriolysins.

With the immune serum on the other hand, results are quite different. Where the most concentrated serum is used, the bacterial growth is still rather profuse; only the moderate doses show a true bactericidal action and the small doses are altogether ineffective. The titer of this serum is between 1/3000 and 1/4000.

Neisser and Wechsberg explain this phenomenon by the so-called "*deviation of the complement.*" They assume that in the serum of higher concentration there are so many amboceptors that the bacteria cannot bind them all. The amboceptors remaining free attach themselves to the complement by means of their complementophile group just as the already bound amboceptors have done. Thus, a part of the complement is deviated from the bacteria and only an incomplete bacteriolysis takes place.

The theory of complement deviation does not in the opinion of the author withstand critical examination. Particularly the evidence brought forward by Bordet and Gengou that the affinity of complement for the bacterium + amboceptor complex (Sensitized bacterium) is considerably greater than for free amboceptor, militates against the view of Neisser and Wechsberg.

It is possible that agglutination may account for the phenomenon of deviation of the complement in that the agglutinated masses of bacteria afford a more resistant barrier to the action of the bacteriolysins. The author has now and then observed an analogous phenomenon in hemolytic experiments; strong doses of hemolysin were less effective than moderate ones, and in these cases the momentary hemagglutination was readily visible. Also, by titrating bactericidal sera in animal experiments, it has been found that moderate doses often afforded the greatest protective action.

For the practical application of the plate culture method, knowledge of the following data is important, as it is necessary to consider the difference between the bactericidal titer of sera of normal and of typhoid patients. According to Korte and Steinberg the bactericidal titer was

	Of normal cases	Of typhoid cases
Under 100 in	74 per cent.	0.0 per cent.
Between 100 and 1000 in	8.6 per cent.	3.3 per cent.
Between 1000 and 10,000 in	15.4 per cent.	15.1 per cent.
Between 10,000 and 100,000 in	2.0 per cent.	23.3 per cent.
Over 100,000 in	0.0 per cent.	58.3 per cent.

The bactericidal titer does not run strictly parallel either with agglutination or the Pfeiffer experiments. It falls toward the end of the disease and is low during convalescence.

Besides being used in typhoid, the plate culture method has been employed for experimental purposes in cholera and dysentery; in these diseases, however, it possesses no clinical diagnostic significance.

Concerning bacillus paratyphosus, the views of different authorities are widely at variance. While some obtained very good results, similar to those found in typhoid fever, Töpfer and Jaffé could demonstrate no bactericidal power whatever in vitro. This difference can be explained only by the differences in sera.

Hemolysins.

An animal that is injected with the red blood cells of a different species, develops in its serum antibodies which are biologically analogous to bacteriolysins and differ from them only in that they cause disintegration of erythrocytes instead of bacteria. These antibodies are therefore called hemolysins, or to be more precise immune-hemolysins, since they arise through a process of immunization. The breaking up of the red blood corpuscle, hemolysis, is recognized by the naked eye. The hemoglobin passes from the erythrocytes into the surrounding fluid (serum or physiological salt solution) and colors it red. The previously opaque blood, lakes and becomes transparent. Immune-hemolysins like bacteriolysins belong to the class of amboceptors. They are relatively thermolabile in that they withstand a temperature of from 56° to 58° C. without being injured, and they require complement for the development of their hemolytic action. Furthermore, immune-hemolysins like all amboceptors, are specific, *i.e.*, the serum of a rabbit immunized against horse's blood can dissolve only the blood of a horse and not that of a hen or cow. On the other hand, group reactions occur here also; for instance the immune-hemolysin produced in a rabbit against horse's blood is likewise active against donkey's blood.

Normal Hemolysin.

Just as various antitoxins, agglutinins, precipitins and bacteriolysins can be found in normal serum, so also normal hemolysins of amboceptor structure can be discovered in the blood of many species of animals or individual animals.

While normal hemolysins come into play in only a few reactions, as in several modifications of the Wassermann test, the significance of immune-hemolysins is extraordinarily great. These antibodies, discovered by Bordet, and independently by von Dungern and Landsteiner, were carefully studied by Ehrlich and Morgenroth and many others. Such researches have, first of all, greatly advanced the subject of immunity in its theoretical aspects, in that they have created the possibility for the discovery in minute detail the finer relationship which has explained some of the phenomena occurring in bacteriolysis. Furthermore, the studies of hemolysins led to the discovery of the complement fixation method, a procedure of exceptional practical value.

Production of Hemolytic Sera.

As far as the technique for obtaining immune-hemolysins is concerned, the rules which hold for every process of immunization are naturally to be followed here also. It is not possible, however, to immunize every kind of animal against every type of red blood corpuscles. Rabbits, goats, horses and chickens are the ones which are best adapted to supply hemolytic sera. An animal produces a better hemolysin the remoter its relationship to the animal from which the erythrocytes for injection are taken. The blood to

be injected, can be employed in just the condition in which it flows from the vein. Nevertheless it is as a rule defibrinated, to prevent coagulation. The simplest and most practical way of doing this is to place some glass beads into a bottle or Erlenmeyer flask and then sterilize by dry heat. The blood coming from the vein is allowed to flow into one of these flasks and then it is repeatedly shaken for several minutes. This suffices to defibrinate the blood and thus prevent coagulation.

The production of hemolysins depends entirely upon the red blood corpuscles. The presence of the serum is not only superfluous, but even harmful, as experience has shown that dangerous reactions may follow the injection of foreign serum.

Washing of Red Blood Corpuscles.

Before injecting, therefore, the erythrocytes are washed. For this purpose a few cubic centimeters of defibrinated blood are poured into a centrifuge tube and the level of the fluid marked on the tube. An equal or double this amount of 0.85 per cent. saline is added, and the tube rapidly centrifugalized. The erythrocytes fall to the bottom, while the upper layers of the tube consist of diluted serum more or less tinged with hemoglobin. The fluid is carefully decanted, fresh saline added, the tube shaken, and again centrifugalized. If this is done two to three times the erythrocytes can be freed of the last traces of serum; finally, by adding saline up to the mark made at the beginning of the experiment the erythrocytes are obtained in the normal concentration, just as in the blood, but completely free of serum.

Immunization.

The injection of the washed, defibrinated blood, can be affected subcutaneously, intravenously, or intraperitoneally. With the subcutaneous and intraperitoneal methods in a rabbit, injections of from 5 to 20 c.c. are necessary at intervals of five to six days. Far larger quantities should be given to bigger animals, like goats and sheep. Subcutaneous injections often cause infiltrations and occasionally abscesses. The author therefore uses the intravenous method exclusively in rabbits.

A suspension of washed blood corpuscles is diluted four to five times with physiological saline; 0.5 to 1.0 c.c. of this fluid is slowly injected into the ear vein every five to six days. Three injections are almost always sufficient for procuring a good serum. The animals sustain the first two injections with ease, but the third and following ones are not altogether without danger. This is supposed to be akin to anaphylactic phenomena. It is therefore advisable to immunize several animals simultaneously, so that in case one dies there is another to replace it. Furthermore there are such marked individual variations in the ability to produce hemolysins that it is best to have several animals to choose from. Beginning on the sixth day after the third injection, blood should be withdrawn for the determination of the hemolytic strength and this process repeated daily until the titer has reached a satisfactory height and then the animal should be bled. If only a small amount of hemolysin is needed, the animal can be allowed to live; it will gradually lose its titer completely and will act apparently like a normal animal. Nevertheless, an essential difference exists. For if the animal previously immunized is again injected, hemolysins reappear after a short incubation period, whereas in a normal animal a *prolonged immunization* is necessary. Hemolysins, therefore, exist to a certain extent

in a preformed state in the cells of an immunized animal. If a stimulus to immunization occurs, the hemolytic substances are thrown off into the circulation, while in a normal animal the formation of hemolysins by the cells must first take place.

The Preservation of Hemolysins.

If a great amount of hemolysin of the same titer is needed, it is best to bleed the animal to death. For the preservation of hemolysins the author recommends the following procedure which he has found very trustworthy. One to 3 c.c. of serum obtained sterile, are poured into sterile tubes, which are closed with absorbent cotton. The tubes are placed into a water bath at 56° C. for one-half hour to inactivate the serum and are then covered with sterile rubber caps. (These are sterilized by placing them in a 1 per cent. sublimate solution for forty-eight hours).

An immune hemolysin must answer both qualitative and quantitative determinations; qualitative, whereby is proven that the serum can dissolve only the red blood cells which serve as antigen or to a slight degree those of nearly related animals, and that it has only the effect of a normal serum upon the erythrocytes of other animals. The quantitative estimation supplies the only means for the absolute differentiation between a normal and an immune serum. In complement fixation where hemolysis bears an active part, it is the quantitative use of the hemolysin which decides the result of the reaction. The immune serum must therefore be "titrated."

If fresh active hemolytic immune serum is used, a constant quantity of blood serving as antigen is mixed with decreasing quantities of serum and the mixtures placed into the thermostat. Results like the following will be obtained.

Antigen blood.	Hemolytic serum of immune rabbit.	Result after 2 hours.
1 c.c. of 5% sheep's blood.....	1 c.c. of active serum, 1 to 10.....	Hemolysis.
1 c.c. of 5% sheep's blood.....	1 c.c. of active serum, 1 to 20.....	Incomplete hemolysis.
1 c.c. of 5% sheep's blood.....	1 c.c. of active serum, 1 to 50.....	Incomplete hemolysis.
1 c.c. of 5% sheep's blood.....	1 c.c. of active serum, 1 to 100.....	No hemolysis.

On the basis of this experiment the titer of the hemolytic serum for sheep's blood would lie between 1/10 and 1/20. But this is incorrect, as it was pointed out previously that by immunization only the amboceptors are increased and the complement remains unchanged. Each of the above dilutions decreases therefore not only the amount of hemolysin, the quantitative estimation of which is the object of the experiment, but also the complement. Inasmuch as the latter was not at first increased, a point is soon reached where there is no complement at all in the diluted fluid; as a result hemolysis cannot occur, for only the combination of hemolysin + sufficient complement, can exhibit any hemolytic action. *Correct titration*

consists therefore in allowing varying quantities of hemolysin with a constant amount of complement to act upon a constant quantity of red blood cells. The simplest method of accomplishing this is first to destroy the complement by inactivation of the hemolytic serum, then to make the desired dilutions, and finally to add to all, the same amount of normal serum as complement. The normal serum of an animal of the same species as that which provided the immune serum can under no circumstances serve as complement. On the contrary, foreign sera are much more suitable; and guinea-pig's serum is especially recommended as complement, when immune rabbit's serum is used. Not every complement serves equally well for any immune serum.

A very good hemolytic system which is almost exclusively used for the complement fixation reaction, is sheep's blood as antigen, rabbit's immune hemolysin as amboceptor and normal guinea-pig's serum as complement. The preparation of these ingredients should be carried out as follows:

1. *Sheep's Blood.*—This should be defibrinated and washed. Washing is necessary because fresh sheep's blood contains complement; and if the blood is a few days old, washing is even more important.

Complementoids.

Although serum which is not fresh does not contain sufficient active complement to cause the danger of superfluous complement, it nevertheless contains substances which interfere with hemolysis. Probably the existence of "complementoids" is the disturbing factor. It must be assumed that complement is composed of two biologically different parts, as is the case with toxins and ferments. One is the haptophore group, which has affinity for the complementophile group of the amboceptor and is the more stable of the two. The other corresponds to the energy group of the toxins (toxophore element) and of the ferments. Just as after the destruction of the toxophore group there remain only innocuous toxoids whose single perceptible activity consists in their ability to neutralize antitoxins, so also, after the destruction of the weakly resistant energy elements of the complement, there remain complementoids which lack the ability to activate a bacteriolytic or hemolytic amboceptor, although by virtue of their uninjured haptophore groups they bind the complementophile groups of the amboceptors. In this way they usurp the place of whatever active complement may still be present, rendering the latter inactive, and as a result hemolysis is absent or incomplete.

Following the technique of Ehrlich and Morgenroth, a 5 per cent. suspension of washed red blood corpuscles is employed to test a hemolysin. A pipette, closed at the top by pressure of the index finger is thrust to the bottom of the washed erythrocytes contained in the centrifuge tube; a definite amount, for instance 1 c.c. is withdrawn and allowed to flow into a graduate. For diluting purposes (in this case up to 20 c.c.) only isotonic or weakly hypertonic NaCL solutions may be used. If water, hypotonic or strongly hypertonic salt solutions are employed, the red blood cells disintegrate. This is not a true biological hemolysis, but depends upon physical basis. 0.85 per cent. saline is most suitable for the majority of erythrocytes (man, rabbit, guinea-pig, ox, sheep). When instead of an isotonic salt solution, an isotonic sugar solution is made, the red cells are retained in their proper

form, but the addition of hemolysin and complement produces no hemolysis. The presence of salt is indispensable for hemolysis as well as agglutination.

Undiluted, unwashed, defibrinated blood if removed sterile can be kept several days in the ice-box. The "Frigo" apparatus is unsuited for this purpose, because the thawing of the frozen blood breaks the capsule of the red blood corpuscle. The deterioration of the preserved blood is recognized by the large hemoglobin content of the serum or the violet color of the blood.

Occasionally blood left in an ice-box becomes dark. This is due to the lack of oxygen. When the 5 per cent. suspension is made and thoroughly shaken, the red color returns. Such blood of course is perfectly serviceable.

Still, it is best not to keep blood longer than four days. Blood older than that, even if apparently unchanged, possesses a lowered resistance and can give a far higher titer in hemolysin tests than fresh blood.

2. The rabbit's hemolysin must be inactivated for one-half hour at 56° C. if it is not kept ready for use in an inactivated state. Dilutions are made with physiological saline.

3. Guinea-pig's complement is obtained by bleeding to death a healthy normal animal.

The blood is allowed to flow directly into a centrifuge tube and then to clot; the clear serum is obtained by centrifugalization. For hemolysin titration it is best to use a constant dose of complement as 1 c.c. of a 1/10 dilution. Complement can be kept for twenty-four hours in the ice-box. When older than this it suffers a distinct decrease in efficiency as complementoid is produced. (See above). In the "Frigo," complement may be kept for weeks. Stern, however, does not recommend complement preserved in "Frigo" for use in complement fixation tests, as its affinity for amboceptor is noticeably decreased.

One c.c. of each of the three reagents (each so diluted with saline that the desired dose is contained within 1 c.c.) is mixed and 2 c.c. of 0.85 salt solution is added to make the total volume up to 5 c.c.

The following controls are absolutely necessary.

1. A test tube showing that hemolysin without complement in strong dosage is ineffective;

2. A test tube indicating that complement without hemolysin in the dosage used is ineffective;

3. A test tube which shows that the NaCL solution is isotonic.

The three reagents must be thoroughly mixed by careful shaking of the tubes which are then placed into the thermostat at 37° C. and hemolysis watched for. The duration of the observation is a matter of personal preference. Only the length of time must always be mentioned. One must say, for instance, that the titer of this hemolysin is 1:800, using 0.1 c.c. of complement under observation for one-half hour, or it is 1:1500 with 0.1 complement under observation for two hours. The time in which hemolysins work is very different. While many hemolysins of the same titer act

in a few moments, others require two hours. The author has made it a rule to read the result after two hours observation, but he notes the progress of the reaction every one-half hour in order to determine whether it is a slowly or rapidly acting hemolysin.

The following chart demonstrates the titration of a hemolysin as a preliminary experiment to the complement fixation method.

	Antigen.	Amboceptor.	Complement.	0.85% Saline	Result of hemolysis. After ½ hr.	After 1 hr.	After 2 hrs.
1.	1 c.c. of 5% sheep's blood	1 c.c. dilution 1 : 10	1 c.c. dilution 1 : 10	2 c.c.	complete	complete	complete
2.	"	1 : 100	"	2 c.c.	complete	complete	complete
3.	"	" 1 : 250	"	2 c.c.	complete	complete	complete
4.	"	" 1 : 500	"	2 c.c.	Incomplete	Almost complete	complete
5.	"	" 1 : 750	"	2 c.c.	Almost 0	Incomplete	complete
6.	"	" 1 : 1000	"	2 c.c.	0	Incomplete	complete
7.	"	" 1 : 1500	"	2 c.c.	0	0	Incomplete
8.	"	" 1 : 2000	"	2 c.c.	0	0	0
Control I	"	" 1 : 10	.....	3 c.c.	0	0	0
Control II	"	.....	1 c.c. dilution 1 : 10	3 c.c.	0	0	0
Control III	"	.....	.....	4 c.c.	0	0	0

Determining the end reaction is a source of difficulty for the beginner. Between the extreme "0" *i.e.*, entire absence of hemolysis, where the appearance of the tube corresponds to that of control III representing a suspension of red blood cells diluted with isotonic saline and the other extreme "complete," *i.e.*, complete hemolysis, where every trace of corpuscular elements has disappeared and a fluid looking like dilute red-wine remains, there are numerous intermediate stages. These intervening grades of reaction are represented by the terms almost 0, incomplete, almost complete, and similar expressions. The meaning of the terms is self-evident. How any particular tube is to be designated is of course a subjective question since the so-called transitional stages are so large in number.

A few hours after the reaction is ended, a remarkable difference may be noted between the tubes in which hemolysis has occurred and those in which hemolysis has been incomplete or totally absent. In the last mentioned, the red blood cells had sunk to the bottom and above them remains a clear fluid which consists of pure saline or diluted serum (complement + immune serum) and is colored accordingly. If the supernatant fluid is richer in hemoglobin than that of the corresponding control, it is evident that some of the erythrocytes were hemolysed and their hemoglobin set free. If the erythrocytes have collected at the bottom apparently in the same quantity as in the

control tube, and form there a large deposit, a trace of hemolysis or almost o would be the terms used in reporting the results. Tube 7 after two hours showed incomplete hemolysis, *i.e.*, compared with control III it was noticeably clearer, but not completely transparent. After twenty-four hours there was a small mass of undissolved red blood cells at the bottom of the test tube and above it a deep red fluid which was only slightly different from that in the tubes where the erythrocytes were completely dissolved. If this sediment should become so small that on shaking only a cloudy turbidity is produced, the result would correspond to the designations "very small sediment," "occasional erythrocytes at the bottom of the tube," or "almost complete hemolysis."

In the tubes containing inactive hemolysin without complement (control 1, and in complement binding reactions) hemagglutination can occur because the agglutinins which also exist in the serum become active. Hemagglutination is recognized by the fact that on shaking the sediment, the erythrocytes are not equally distributed, but remain in clumps or strings and soon sink to the bottom again.

Titration of the Complement.

For many purposes it is desirable to titrate the complement content of a serum. The method is the same as that used in hemolysin titration, only with the difference that a fixed amount of hemolysin and varying quantities of complement are employed.

	Antigen.	Amboceptor.	Complement.	NaCl. solution.	Result after two hours.
1.	1 c.c. 5% of sheep's blood.	1 c.c. hemolysin dil. 1:1000.	1 c.c. 1:10 (= .01).	2.0 c.c.	complete.
2.	1 c.c. 5% of sheep's blood.	1 c.c. hemolysin dil. 1:1000.	0.8 c.c. 1:10 (=0.08).	2.2. c.c	complete.
3.	1 c.c. 5% of sheep's blood.	1 c.c. hemolysin dil. 1:1000.	0.6 c.c. 1:10 (=0.06).	2.4 c.c.	complete.
4.	1 c.c. 5% of sheep's blood.	1 c.c. hemolysin dil. 1:1000.	0.4 c.c. 1:10 (=0.04).	2.6 c.c.	complete.
5.	1 c.c. 5% of sheep's blood.	1 c.c. hemolysin dil. 1:1000.	0.2 c.c. 1:10 (=0.02).	2.8 c.c.	Incomplete.
6.	1 c.c. 5% of sheep's blood.	1 c.c. hemolysin dil. 1:1000.	1.0 c.c. 1:100 (=0.01).	2.0 c.c.	o
7.	1 c.c. 5% of sheep's blood.	1 c.c. hemolysin dil. 1:1000.	—	3.0 c.c.	o
8.	1 c.c. 5% of sheep's blood.	—	1 c.c. 1:10 (=0.1)...	3.0 c.c.	o
9.	1 c.c. 5% of sheep's blood.	—	—	4.0 c.c.	o

The titer of this complement when employed with a hemolysin of 1/1000 strength and allowed to stay in the incubator for two hours would be 0.04.

The complement content of the serum of a healthy guinea-pig is fairly constant. During illness the titer usually is decreased. Among healthy people the complement titer shows marked individual variations.

For hemolysis a definite quantitative relationship between hemolysin and complement is necessary.

On the basis of the two titrations outlined above, it has been estimated that at least 0.04 c.c. complement is necessary to activate 0.001 c.c. of hemolysin. If less complement is used with the same amount of hemolysin, hemolysis does not occur or else it is incomplete. If the quantity of hemo-

lysin is increased for instance threefold, then it will be found that 0.02 c.c. of complement suffices to produce hemolysis. Vice versa with an excess of complement the hemolysin titer of 0.001 c.c. may be reduced. However, there are narrow limits to this mutual compensatory action.

Cytotoxins, Cytolysins.

The hemolysin bodies are characteristic and important members of a general class of substances known as cytotoxins, especially investigated by Metschnikoff and his co-workers.

Just as the immunization with erythrocytes led to the production of lytic amboceptors which in connection with complement destroyed and dissolved their antigens, so in a similar manner, various substances more or less specific for their antigens have been produced through immunization with leucocytes "Leucocidin;" with nerve tissue, "neurotoxin," with spermatozoa, "spermatoxin," and kidney tissue, "nephrotoxin." The proof of their action, particularly of neuro-, nephro-, and hepatotoxin is not simple. As all these cytotoxic sera have at the same time a hemolytic action, it is not easy to decide to what extent the changes in the organs observed after the injection of the cytotoxic substances are dependent upon the action of hemolysins. It must further be taken into consideration that none of these sera are absolutely specific for the organ in question. This is not surprising, inasmuch as there are widespread common group characteristics (common receptors) among the different organs serving as antigens, and only very few groups of a specific nature. The hopes which, at the beginning, were placed upon the study of cytotoxins particularly with the expectation that they would tend to become diagnostic and therapeutic methods for the treatment of malignant tumors, have as yet been unrealized. The entire field of cytotoxins urgently requires further investigation.

CHAPTER XIII.

The Method of Complement Fixation.

Its principle, antituberculin, Ehrlich's side-chain theory, serum diagnosis of syphilis, and diseases caused by animal parasites.

The Question of Multiplicity of Complements.

It has already been demonstrated that neither bacteriolysis nor hemolysis can take place without the presence of complement. The question therefore arises whether this complement is the same in both of these reactions or whether normal serum possesses different complements. In order to solve this, a number of very complicated experiments have been carried out by Ehrlich and Morgenroth, Metschnikoff and Bordet and Gengou. Ehrlich and Morgenroth endeavored to show that not only do the complements of different animals of the same class vary, but that numerous complements exist within one individual serum (conception of the multiplicity of complements). Metschnikoff believed that each serum contained at least two complements, the microcytase and the macrocytase, thus enlisting the supporters of a dualistic theory. Bordet and his school, on the other hand, although agreeing with the idea that the complement varies in different animals, deny its multiplicity and contend that any given serum contains but one alexin, or complement—the theory of unity of complement. It would be superfluous to cite all the experimental data supporting these opinions, but nevertheless a review of the classical experiment of Bordet and Gengou which corroborated the existence of only one complement, thus offering the fundamental principle for the establishment of the most important method of serum diagnosis, namely, complement fixation, would not be out of place.

The Principles of Complement Fixation.

Bordet and Gengou mixed in a test-tube typhoid bacteria (antigen), inactivated typhoid immune serum (amboceptor) and normal serum (complement). Union of the bacteria and immune serum first took place followed by absorption of, and coalescence with, the bacteriolytic complement contained in the normal serum. As a result, bacteriolysis occurred and the bacteriolytic complement was used up during this process. Bordet and Gengou reasoned that if the bacteriolytic and hemolytic complements were identical, then in the above mixture of typhoid bacteria, immune serum and normal serum, the hemolytic as well as bacteriolytic complement should be absent, while

if the plurality of complement exists, the hemolytic complement should still be present. Accordingly, after a certain interval, washed erythrocytes and inactivated homologous immune serum were added and hemolysis looked for. No hemolysis took place, thereby attesting to the fact that the bacteria in the first part of the test had "fixed" ("held in check") not only the bacteriolytic but also the hemolytic complement. Bordet and Gengou thereupon named this test "complement fixation" or "complement binding" (La fixation d'alexine).

With the aid of this experiment Bordet and Gengou were able to prove a number of theoretically important points. They demonstrated among other things *that absorption of complement was not always necessarily accompanied by bacteriolysis.* For example, the anthrax and pest bacteria when mixed with their respective homologous immune sera show no or only very incomplete bacteriolysis. The erroneous conclusion thus reached to the effect that these sera contained no amboceptors, was disproved by Bordet and Gengou, who demonstrated that, 1. these sera contained amboceptors in spite of the absence of bacteriolysis, 2. the complement was absorbed, although no bacteriolysis took place.

During the process of immunization, amboceptors were found far more frequently than bacteriolysins. These two terms must not be considered as synonymous.

Amboceptor signifies a more generic term, and one must differentiate between amboceptors of cytolytic and non-lytic properties. Whether the difference here really depends upon the different nature of the amboceptor, or upon the construction and constitution of the antigen, is not solved.

The fixation of the complement, precedes the act of bacteriolysis. The important requirement for the fixation is an antigen which has been sensitized by the attachment of the amboceptor, thus increasing the affinity toward the haptophore group of the complement. Antigen alone, or even amboceptor alone, cannot or perhaps only very slightly bind the complement. Whether the zymotoxic (energy) group of the complement manifests its activity (bacteriolysis) or not (absence of bacteriolysis) is materially indifferent for the complement fixation.

Complement Fixation as a Method of Serum Diagnosis.

Through complement fixation, as introduced by Bordet and Gengou, one is enabled to prove the presence of specific antibodies when the antigen is known or reversely, an unknown antigen provided the specific antibody is given. This method of serum diagnosis can be widely employed, as the majority of bacteria and immune sera (with the exception of pure antitoxic sera)[1] when mixed homologously, give a positive reaction—the absence of hemolysis, proving the absorption of complement by the union of the antigen and its specific amboceptor. This reaction is strongly specific. If bacteria are mixed with an inactive heterologous immune serum, or with a heated normal one, not in concentrated form (normal amboceptor), and complement is added, the latter will not be fixed but remains to be taken up by the subsequently added red blood cells, and its immune serum, causing hemolysis. Hemolysis indicates that the mixed bacteria and serum are not

[1] Even antitoxic sera are said by Nicolle to give complement fixation reactions.

homologous, as the complement is left free, and given a chance to unite with the added erythrocytes and hemolytic amboceptor. In the case where the bacteria are known, *e.g.*, typhoid bacilli, the occurrence of hemolysis indicates that the examined serum contains no typhoid amboceptors. If the serum is known (*e.g.*, meningococcus serum) the occurrence of hemolysis proves that the bacteria under examination are not meningococci. The absence of hemolysis, will in the first case point out that the unknown serum contains typhoid amboceptors, *i.e.*, is a typhoid serum; while in the second case the

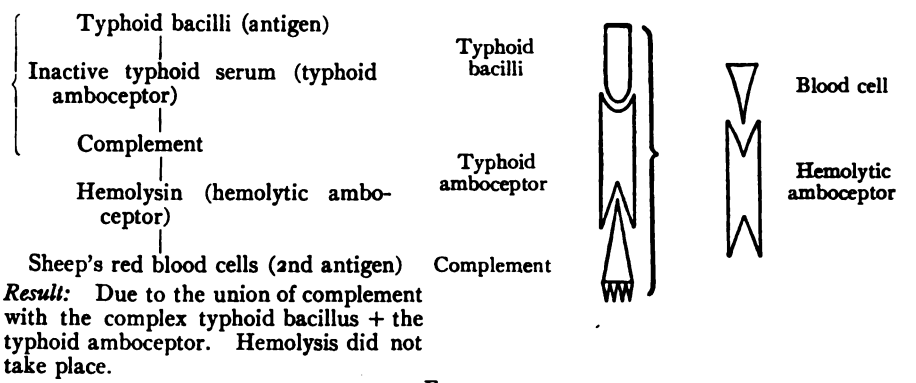

FIG. 15.

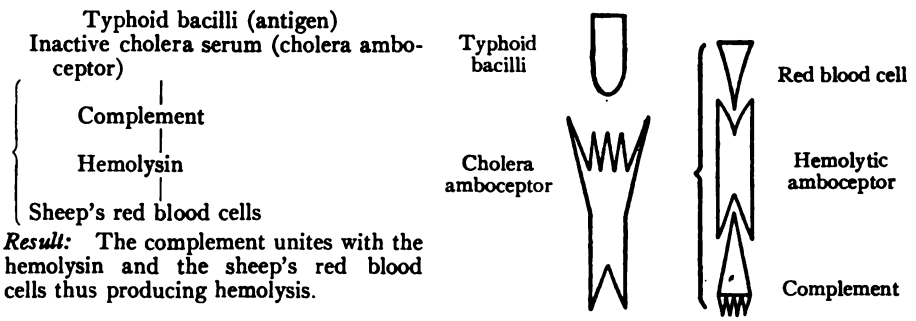

FIG. 16.

absence of hemolysis would bear definite evidence in favor of meningococci. The accompanying figures, 15 and 16, represent schematically, the positive and negative complement fixation test.

Gengou further showed that not only cellular antigens can stimulate the formation of amboceptors, but that during the course of immunization with proteids in solution (milk, serum, etc.), complement binding amboceptors are also formed in addition to the precipitins. Citron has therefore proposed the term "antigenophile," to designate the "cytophile" group of the amboceptor.

Widal and Lesourd, were the first to make practical application of the complement fixation property. They found that the Bordet-Gengou reaction could be obtained far more frequently and earlier with the serum of typhoid

patients than the agglutination test. Nevertheless, this entire complement fixation method remained unheeded for several years.

Moreschi (at Pfeiffer's institute), while conducting some theoretical studies concerning the nature of anticomplements, *i.e.*, such substances which tend to neutralize the action of complements, discovered anew, that by the mixture of a soluble proteid with its antiproteid serum the existing complement disappeared. This, as has been seen, can be explained by the presence within the immune serum, of bodies similar to Gengou's amboceptors. Moreschi, however, stated that the complement disappeared because it was thrown to the bottom mechanically, by the occurrence of precipitation. Such a physical explanation for the complement fixation reaction lead a number of authorities to the belief that the positive Bordet-Gengou reaction was in reality no amboceptor action, but a result of a similar precipitation process. Wassermann and Bruck, Liefmann, Wassermann and Citron, and later on Moreschi himself realized that this physical explanation was incorrect, inasmuch as complement fixation took place even if all precipitation was prevented by heat or other influences. Furthermore, complement binding of an unspecific nature can be produced by the mixture of glycogen or peptone with serum, a procedure wherein surely no precipitation plays any part. Finally Moreschi showed that there were strongly precipitating sera which nevertheless did not exhibit the Bordet-Gengou phenomenon.

Thus was definitely established that the complement fixation was entirely independent of either bacteriolysis or precipitation.

Following Moreschi's researches, Neisser and Sachs continued Gengou's studies and advised this demonstration of the proteid amboceptors as a control to the precipitation method for the differentiation of proteids. Its action is so much finer, and more delicate than the precipitin test that even the minutest traces of proteid can be recognized.

With the encouraging results of Neisser and Sachs in mind, Wassermann attempted by the use of highly immune antibacterial serum to discover any soluble bacterial proteids which may exist in the blood, derived from the respective bacteria invading the organism at the onset of an infection. Practical application proved that not enough of these proteids existed free in the circulation, but that they were probably bound by the tissue cells.

Wassermann and Bruck then employed the complement fixation test with the idea of demonstrating the existence of the respective antigens in the diseased organs. Tuberculous glands and lungs served as material for this experiment. They were able to obtain complement fixation when an extract of tuberculous organs as antigens was mixed with a tuberculous serum (manufactured by the Höchst Farbwerke). If instead of the latter, the serum from tuberculous individuals was substituted, no positive complement fixation reaction was obtained. On the other hand, the reaction was given if the human tuberculous serum employed came from an individual who had received therapeutic inoculations of tuberculin. In other words, the serum of treated individuals contained, in contrast to the untreated ones, amboceptors against a soluble tuberculous substance also present in the

Antituberculin.

extract of tuberculous glands. Wassermann and Bruck identified this substance as tuberculin, because the sera of the treated individuals gave the same positive results if a solution of old or new tuberculin was used instead of the extract of tuberculous organs. Thus, the latter contained tuberculin while the sera of the tuberculin-treated individuals contained amboceptors designated by Wassermann and Bruck as "antituberculin." The name antituberculin has not been a well chosen one, because it creates the impression among many as being an antitoxin. It is better to speak of it as antituberculin amboceptors.

Since, according to Wassermann and Bruck these antituberculin amboceptors were not supposed to be formed spontaneously in tuberculous individuals, but only in those treated with tuberculin, their demonstration could be of no apparent diagnostic value. On the other hand, their existence greatly furthered the understanding of Koch's tuberculin reaction, as most tuberculous individuals who had antituberculin amboceptors in their serum did not respond to the subcutaneous injection of tuberculin.

Wassermann and Bruck, moreover, showed that a mixture of tuberculin with an extract from tuberculous organs bound complement. From this they concluded that the extract likewise contains antituberculin amboceptors. Thus reasoning they developed their tuberculin theory.

Tuberculin Theory of Wassermann and Bruck.

The difference in the reaction observed in a normal and tuberculous individual after inoculation of tuberculin, can be fully explained by the presence of antituberculin amboceptors in the tuberculous focus. By virtue of their specific affinity, the amboceptors attract the injected tuberculin toward them. The tuberculin and antituberculin unite, and absorb the complement from the circulating blood stream, since the complementophile group of the amboceptor is free and unbound. By virtue of the fresh complement which is an actively lytic ferment, and the attracted leucocytes, a partial destruction and casting off of the tuberculous focus results. Upon this depends the therapeutic effect of the tuberculin. During a prolonged treatment with tuberculin, the body produces an excess of antituberculin amboceptors so that finally some appear free within the blood serum. When this is the case the tuberculous organism loses its power to react toward tuberculin, as the latter is neutralized in the blood-stream at a point away from the local focus. No therapeutic effect is any longer obtained from the tuberculin injections, so that they can, for a time, be suspended. The aim of tuberculin therapy should be to work with small doses so that only a focal reaction is obtained and the hyperproduction of antituberculin amboceptors be postponed as long as possible.

Numerous exceptions were at once taken to this theory and its experimental data, the most important of which can here be mentioned.

"Summierung's Einwand" (Exception taken on Ground of Summation of Antigen.)

Weil and Nakayama disagreed with the proof of the existence of "antituberculin" in the organ extracts, on the basis that Wassermann had overlooked the effect of a summation of antigen. This is best explained as follows: Complement is bound not only by antigen + amboceptor, but also by large doses of antigen itself dependent upon the normally present amboceptors existing in the serum employed for complement.

Old tuberculin.	Complement.	Erythrocytes.	Hemolysin.	Result.
0.05	0.1	1 c.c. 5%	Twice the hemolytic titer.	Hemolysis.
0.1	0.1	1 c.c. 5%	Twice the hemolytic titer.	Hemolysis.
0.15	0.1	1 c.c. 5%	Twice the hemolytic titer.	No hemolysis.

0.15 old tuberculin is thus sufficient of its own accord to bind complement. In their experiment, Wassermann and Bruck found that,

Tuber-culin.	+ Extract of tuberculous organs.	Complement.	Erythrocytes.	Hemolysin.	Result.
0.1	0.1	0.1	1 c.c. 5%	Twice the hemolytic dose.	Complement fixation.
0.1	.....	0.1	1 c.c. 5%	Twice the hemolytic dose.	Hemolysis.
....	0.1	0.1	1 c.c. 5%	Twice the hemolytic dose.	Hemolysis.

This, however, in no way proves the existence of "antituberculin" in the extract of tuberculous organs, as it is perfectly possible and even probable that 0.1 of the organ extract contains 0.05 c.c. at least of tubercle bacillus substance (tuberculin) which when added to 0.1 of tuberculin used for antigen, is sufficient to give an amount of tuberculin perfectly capable, as has been seen, of binding complement by its own activity.

In order to overcome this possibility one must work with such small but at the same time maximum amounts of antigen and antibodies, that at least double the quantity of each of these reagents does not, of its own accord, bind complement. For tuberculin this is estimated as follows:

Tuberculin.	Complement.	Hemolysin.	Erythrocyte.	Result.
0.2	0.1	2×Hemolytic dose	1 c.c. 5%	No hemolysis
0.18	0.1	2×Hemolytic dose	1 c.c. 5%	No hemolysis
0.15	0.1	2×Hemolytic dose	1 c.c. 5%	No hemolysis
0.14	0.1	2×Hemolytic dose	1 c.c. 5%	Incomplete hemolysis
0.12	0.1	2×Hemolytic dose	1 c.c. 5%	Complete hemolysis
0.1	0.1	2×Hemolytic dose	1 c.c. 5%	Complete hemolysis

Twelve-hundredths is the maximum non-binding or hemolytic dose. For the complement fixation test where the object is to demonstrate anti-tuberculin amboceptors, the maximum amount of antigen to be used is therefore 0.06 T. or one-half of the maximum non-binding dose.

In the same way the hemolytic dose, and the antibody containing reagent, should be estimated.

Organ extract.	Complement.	Hemolysin.	Erythrocyte.	Result.
0.2	0.1	2×Hemolytic dose	1 c.c. 5%	0
0.16	0.1	2×Hemolytic dose	1 c.c. 5%	Complete hemolysis
0.1	0.1	2×Hemolytic dose	1 c.c. 5%	Complete hemolysis

The non-binding dose is 0.16. The amount, however, to be employed in the complement fixation test must be 0.08 c.c. of organ extract.

If on mixing 0.06 T. and 0.08 extract, complement fixation still appears, then this summation of antigen can be disregarded and an antigen antibody reaction must be considered. For, even granting that 0.08 of extract does for its greater part, *e.g.*, 0.06 at the most, contain tuberculin, then this amount + 0.06 of the tuberculin in the antigen only makes 0.12 of tuberculin, a quantity not sufficient to fix the complement. De facto, complement fixation does occur when the above test is carried out with proper dosage, so that most probably it is occasioned by the biological antigen antibody reaction. *As a general rule for all complement fixation tests, the dose of each ingredient employed should never be more than 1/2 of its maximum complement non-binding quantity.*

This principle was not definitely established at the time of Wassermann and Bruck's first studies so that experimental proof for the existence of antituberculin amboceptors in tuberculous organs has not been corroborated.

Other Exceptions.

A second exception, taken by Weil and Nakayama as well as by Morgenroth and Rabinowitsch relates to the activity of the complement when it combines with tuberculin and antituberculin.

They claim that by this union the complement's lytic function is entirely lost. Morgenroth and Rabinowitsch even go so far as to deny the existence of antituberculin in the blood of tuberculous individuals.

The author also undertook a minute study of this question and came to the following definite conclusion.—There are some tuberculous individuals who spontaneously develop antituberculin amboceptors, a fact to be expected because it has for a long time been known that on and off tuberculin can be liberated in the organism of tuberculous individuals. As a natural consequence antibodies will be formed, and most probably by those tissue cells in the neighborhood of the liberation of the tuberculin, *i.e.*, the focus of infection.

Before proceeding, however, to the author's conception of the tuberculin theory it is necessary to review Ehrlich's principles of immunity upon which the ideas of antibodies and their specificity are based.

Ehrlich's Side Chain Theory.

Ehrlich's idea of the biological structure of cells is that they consist of two parts, a central functionating radicle ("Leistungskern") upon which depends the specialized activities of the cells, as for example, a glandular or nerve cell, and a multiplicity of side chains or receptors (a term borrowed from the chemistry of the benzol group), by means of which the cell enters into chemical relation with food and other substances brought to it by the circulation. These receptors are exceedingly numerous, as the nutritive substances upon which the cell depends for its maintenance are very varied. Besides these general receptors the special cells also have different and special side chains; then, too, there exist very great quantitative differences among the latter; and finally it must be added that the selective activity of the cells depends upon the variability of these receptors.

When an infection occurs, pathological material is brought to the cell bodies instead of physiological normal substances. Certain of these poisonous products find suitable receptors in all of the cell groups, others fit only into distinct groups of cells, while a third class are not taken up at all. This is strikingly in evidence, for the organism which possesses no receptors for any of the pathological agents, cannot assimilate any deleterious substances and is therefore immune. Lack of amboceptors is therefore a natural form of immunity. The organism having only a special group of cells for the reception of certain pathological matter, will make use of these cells for the binding and assimilation of the toxic material. For example, the nerve cells alone have receptors for tetanospasmin; no matter how or when the poison is introduced into the organism the nerve cells will absorb it. As this toxin is poisonous for the central atom group (Leistungskern) of the nerve cell, the latter is destroyed. The union between the nerve cell receptors and the tetanospasmin toxin is only the preliminary act for the cell destruction; the actual death of the cell being caused by the action of the toxophore group of the poison upon the functional radicle of the cell. If, however, such receptive side chains are possessed not only by the brain but also by other cells, *e.g.*, connective tissue cells, the tetanospasmin will in part be bound by the latter. The toxophore group of the toxin does not have any harmful effect upon the functional radicle of these cells, and thus no toxic effects will be incurred by the union, and the nerve cells remain unaffected.

The number of receptors which cells possess for tetanospasmin, for example, are limited and after their junction with the teanospasmin, are rendered useless and inactive. By the normal reparative mechanism of the body, new receptors are generated. This reparative process does not as a rule stop at a simple replacement of lost elements, but according to the hypothesis of Weigert tends to overcompensation. The receptors eliminated by toxin absorption are reproduced in an excess of the simple physiological needs of the cell. Continuous and increasing dosage of the toxin soon leads to such excessive production of receptors that the latter find no more room to be attached to the cell, but are cast off and circulate free in the blood. They still, however, retain their property of being able to combine with tetanospasmin.

If such an organism is injected with tetanospasmin the latter toxin is bound by the free receptors in the serum, and thus the respective "sessile" receptors attached to the cells are precluded from coming in contact with the poison. Inasmuch as the free receptors possess no functional radicle which can be injured, the toxin remains entirely innocuous for the individual. Such protective bodies lend to the organism its attained immunity and are known as antitoxins. Their function can be compared to lightning rods.

v. Behring well expresses their action when he states that the same elements which attached to the cells render the body susceptible to toxic substances, when circulating *freely in the blood serve* to protect it.

The antibodies against toxins and ferments are of the simplest form. They possess only a binding group which has an affinity toward the haptophore group of the toxins and ferments. They, therefore, belong to the class designated by Ehrlich as "haptines" of the first order.

To the haptines of the second order belong the agglutinins and precipitins. They possess besides a haptophore group also an agglutinophore or percipitinophore group by virtue of which agglutination or precipitation takes place.

Belonging to the haptines of the third order are the class of amboceptors which have in addition to the haptophore group also a complementophile group for their union with the complement.

These hypotheses of Ehrlich greatly simplify the explanation of many serum eactions as well as many of the phenomena associated with the action of tuberculin. In all probability the healthy cells which exist in the tuberculous focus and which are capable of reaction, produce the antituberculin. Christian and Rosenblatt offered experimental evidences for this statement. They demonstrated that tuberculous guinea-pigs in whom antituberculin was produced by tuberculin injections, showed a diminution of antituberculin in the blood when tuberculous glands were removed by operation.

The antituberculin production by the cells is a transitory action arising only when tuberculin has spontaneously or artificially reached the circulation. Following this stage of activity there comes a period of quiescence during which no free antituberculin can be found in the serum. The cells, however, are supplied with a great many more sessile receptors than usually; they possess a higher affinity toward tuberculin and produce antituberculin much more readily than normal cells.

This also explains why the smallest amounts of tuberculin produce a reaction in tuberculous and not in the normal individuals. In the former, the cells in the zone surrounding the tuberculous focus are abundantly supplied with receptors, so that on the injection of tuberculin, its action appears almost concentrated at this point. Occasionally the sessile receptors are relatively scarce and the first injection excites no reaction. By the time of the second or third inoculation these sessile amboceptors have so increased that a positive reaction is apparent when the same or even a smaller dose is injected. This phenomenon of increased sessile receptors explains the reappearance of subsided, subcutaneous, cutaneous, or ophthalmo reactions after renewed injections of tuberculin.

To recapitulate the biological phenomena associated with a positive tuberculin reaction, it may be said that the tubercle bacilli, or portions of their body substances existing in the infected focus, stimulate the adjacent cells to produce a great number of sessile receptors. When the tuberculin is injected for the first time, these sessile receptors at once take up the tuberculin and as a result, the production of antituberculin in the focus is further stimulated. Every production of antibodies, is, if the stimulant be strong enough, associated with fever; in this very regard, however, Wright as well as Pfeiffer and Friedberger showed that if the smallest doses of antigen are employed, antibody production continues without any rise in temperature. Fever in a tuberculin reaction is therefore not a necessary manifestation of a positive tuberculin reaction, although it generally is present. The enlarged number of sessile antituberculin receptors augments the affinity of the cells toward the tuberculin, and the second, third and succeeding inoculations bring about a focal reaction (*i.e.*, antituberculin production) much more

easily. Finally the antituberculin receptors become so numerous that they are detached from the cells and become free receptors. This period, however, is only transitory, as is corroborated by the difficulty connected with the demonstration of these antibodies in the focus. This free antituberculin combines with the tuberculin (spontaneously formed or injected) and attracts the complement, or the complement producing phagocytes. Uncombined complement has no effect on the tissues. It is different, however, with the phagocytes. These can without any additional help act directly upon the infected focus. If the tuberculin treatment is continued, a period arises during which the antituberculin bodies are so greatly accumulated in the local focus that they ultimately escape into the blood stream. This freely circulating antituberculin neutralizes any freshly injected tuberculin, so that such patients become refractory even against the largest amounts of it. (Tuberculin immunity.) Tuberculin immunity is not, however, in all cases to be identified with a strong antituberculin content in the serum. For example, it is very difficult to stimulate antituberculin by treatment with S. B. E., although by its use an immunity against B. E. is easily attained.

In former times a negative tuberculin reaction after a prolonged treatment was stamped as a cure of the tuberculosis, a fact obviously incorrect; for, no matter how successful the tuberculin therapy may be, it cannot be considered as a complete curative procedure.

The appearance of antituberculin in the general circulation is interpreted in a double light. Wassermann and Bruck advised that it was best to avoid its appearance, because by its presence here the tuberculin is neutralized without ever reaching the focus where it is required. On the other hand, it may be considered a protective element in that it binds any tuberculin which may spontaneously be formed in the system. *In general, that method should be adopted which makes the subject non-susceptible to the largest doses of tuberculin.* In practice it was found that those patients having the greatest amounts of antituberculin in their serum, generally offered a better prognosis than the others.

Recent experiments of the author seemed to show that in certain cases serum containing antituberculin can raise the susceptibility for tuberculin. Thus tuberculous guinea-pigs injected with a mixture of tuberculin, antituberculin and complement in proportionate dosage, died in several hours, while animals of the same kind receiving only tuberculin or tuberculin + antituberculin remained alive.

Serum Diagnosis of Syphilis.

The experiences gained by the employment of the complement fixation test in tuberculosis, lead to its application in the study of syphilis. The difficulties in this disease were greater, inasmuch as there were no bacteria or preparations like tuberculin which could be used as antigen.

Syphilitic organ extracts were employed instead, with the idea that these would contain the specific virus. The serum of monkeys previously immunized with such extracts when mixed in vitro with the latter, gave complement fixation. This experiment is not, however, conclusive as the positive reaction may be due to anti-human proteid amboceptors produced at the same time by the injection of the human serum *contained in* the organ extract. The experiment was changed and the syphilitic organ

extracts from apes were used so as to exclude the error. Even in this way complement fixation was attained. Later on it was found unnecessary to inject the monkeys with the extracts since after ordinary infection their serum would give complement fixation. In this manner it was almost definitely established firstly, that these extracts contained a substance specific for syphilis which could with most probability be considered a luetic antigen, and secondly that infected apes possessed antibodies against this antigen.

The next step was to try the reaction in man. The first experiments of Wassermann, Neisser, Bruck and Schucht did not give the hoped for returns. Although the reaction was obtained with human serum, the percentage of positive results was so small (see next chart) that its practical value as a means of diagnosis offered no great help. Only in general paralysis did the expectation seem promising. In about 80 per cent. of all cases Wassermann and Plaut were able to demonstrate the luetic antibodies in the cerebrospinal fluid.

Schütze's experiments in tabes led him to the same findings. Citron has obtained a much smaller percentage of positive reacting cerebrospinal fluids in tabes.

As it seemed that the means of diagnosis was not to be established by the demonstration of the syphilitic antibody, Neisser and Bruck believed that better results may possibly be achieved by the discovery of the luetic antigen in the serum through complement fixation.

This attempt too was unsuccessful. No antigen could be found, but the extracts of red blood cells from syphilitic individuals when mixed with the serum of highly immunized monkeys gave a positive complement fixation. Neisser and his co-workers concluded therefrom that the erythrocyte extract contained the luetic antigen. Citron soon demonstrated that the extracts of normal individuals gave a similar reaction and what was more important, that this so-called blood antigen existed in the blood entirely uninfluenced by mercurial treatment. Since these experiments, not much importance has been attached to this reaction.

Meanwhile the author working at the Kraus clinic proved by a large series of experiments that luetic antibodies were present in almost all cases of lues. The reaction is dependent upon two rules.

The First.—The longer the syphilis virus has acted upon the organism and the more numerous its recurrent manifestations have been, the more frequently will a positive reaction be obtained and the stronger will the antibody content of the serum be.

The Second.—The sooner a proper mercury therapy is instituted, the more often it is repeated, and the shorter the interval since the last treatment, the smaller will the antibody content of the serum be and the greater the possibility of a negative reaction.

These points were soon corroborated by numerous other workers in the field, so that at the present day, they can be taken as absolute facts. The following chart will explain some of the statements aforementioned.

	First period.					Second period.							
						Citron.							
	Wassermann, Neisser, Bruck & Schucht.	Wassermann and Plaut.	Marie and Levaditi.	Morgenroth and Stertz.	Schütze.	1st. report.	2nd. report.	3rd. report. (with Blaschko.)	Bruck and Stern	Fleischmann.	Meier.	Ledermann.	Micheli and Borelli.
	%	%	%	%	%	%	%	%	%	%	%	%	%
Lues I	8	.....	.....	.....	.....			90	48.2	100	68	52.6	....
Lues II	26.7	.....	.....	.....	.....			98	79	93	93	100	92.8
with symptoms.													
without symptoms ("early latent")	14.6	.....	.....	.....	.....			80	20	64		75.6	46
Lues III	....	.....	.....	.....	.....	77.5	74	....	....	...	...	.....	....
with symptoms	21.6	.....	.....	.....	.....			91	57.4	98	100	92.2	88.9
without symptoms ("late latent")	11.3	.....	.....	.....	.....			57	20.2	42	...	36.8	37.5
Progressive paralysis	....	(80) (spinal fluid)	(75) (spinal fluid)	(100) (spinal fluid)	.....	100	.....	...	...	...	...	88	....
Tabes dorsalis	....	.....	.....	.....	(66) (spinal fluid)	86.6 (22) (spinal fluid)	79	60	....	...	...		

As has been repeatedly remarked, *specificity* is the important element in every biological reaction. The reaction known after the discoverer as the Wassermann Reaction, can also be performed if instead of the extract from luetic organs, an alcoholic extract of certain normal organs or certain lipoid substances is substituted as antigen. Seligmann too, was able to obtain complement fixation by pure chemical reactions. Consequently, numerous authorities expressed the opinion that the Wassermann Test was non-specific and that it does not at all represent an antigen antibody interaction.

There is no doubt, however, that this exceptional view is incorrect. It is true that the *real* syphilitic antigen is unknown, but most probably it is neither the pure spirochætes

nor a pure lipoid substance. The author has expressed the hypothesis to the effect that the antibody producing antigen is a toxolipoid. This explains the fact why pure lipoids can stimulate no antibodies, but can at the same time react with luetic antibodies in vitro.

The accompanying diagram (Fig. 17) explains this hypothesis. In order to answer the objections raised against this theory, the author has proposed the indifferent term of "Lues-reagine" for the luetic antibodies as long as their biological structure is unknown.

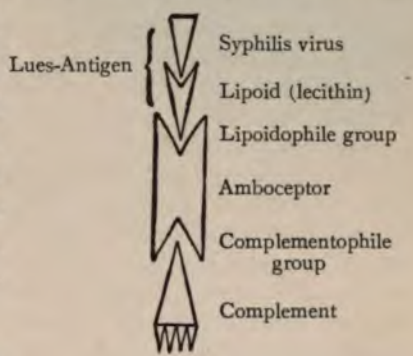

FIG. 17.

Independent of the question of "biological specificity," the Wassermann reaction must also be considered in the light of a "clinical specificity." From this standpoint it fulfills its demands. With only few exceptions, it can be considered absolutely specific for lues.

The well established exceptions are, frambœsis, trypanosomiasis, leprosy, malaria, scarlet, febris recurrens. The reactions obtained here are similar, but not the same as those obtained in syphilis. In leprosy the point of difference is seen in that the reaction can also be performed with tuberculin as antigen; in scarlet the reaction appears only in a small percentage of cases and not with all luetic extracts. Furthermore, it disappears at the latest three months after the infection, usually much sooner. As for trypanosomiasis and malaria convincing data are still too few.

These diseases excluded, a positive Wassermann reaction can be taken as certain proof for the existence of lues. Whether such a test is indicative of a by-gone infection or whether it means that an active process is still going on at the time of its obtention has been for a long time a subject of discussion. The author is of the firm opinion *that the demonstration of the "lues reagine" means active lues.* The reasons for this belief are as follows:

1. The almost constant presence of the reaction in all cases of manifest lues excepting primary lesions. During this stage it is entirely absent or only partly detected. It appears, however, later on.

2. The practically assured existence of the reaction with a recurrence of symptoms even if before that the reaction was negative.

3. The possibility of influencing a positive reaction so that it becomes negative, by the use of mercury. The latter holds true also for those cases which show no symptoms and are therefore incorrectly designated as latent syphilis. It has been proven that such are in reality by no means latent, but have an active process at some point escaping detection, as the aorta. Only cases of a nature which have no symptoms and a negative reaction should be considered as latent syphilis, those however with no symptoms, but a positive reaction as belonging to the class of active lues.

4. The evidence that apparently healthy individuals, but with a positive

reaction, have infected others or have all of a sudden developed tertiary or postluetic manifestations, tabes, paralysis, diseases of the aorta, etc.

An objection has frequently been raised, that in spite of existing disease, the reaction has been found negative. If the statistics covering the largest number of cases are studied, it will be seen that such instances are rare. Few exceptions are discovered in every biological reaction, especially one which is complicated and where five different ingredients come into play; even in the immunization of animals differences will be found in that some produce a highly agglutinating or precipitating, etc., serum, while others will show few or even no antibodies. Individual differences are prevalent to such extent that exceptions to the rule must be taken for granted. Fortunately, a negative reaction in existing lues is so rare, that for practical purposes its possibility may be overlooked, at least, with reservation.

As a general rule, antibodies persist in an organism for a certain time past infection, when the individual has become perfectly well. Exceptions, to the effect that it may be possible for a positive Wassermann reaction to similarly signify a past infection or a state of immunity, have been raised. But it must be said that immunity in syphilis is a condition thus far unproven and almost unknown. All symptoms previously attributed to such an immunity can more easily be explained in the light of a continuation of the disease. As for the "lues reagine" remaining after the cure of the infection, undoubtedly this phenomenon is possible. The analogy with other diseases seems lost, however, when one considers that the syphilitic reaction is discovered thirty or forty years after an infection, while antibodies in general persist for weeks, months or at the most for several years, following an infection. Still it may be possible that the syphilis "reagine" is characterized by the difficulty with which it is excreted and by the tendency of the cells when once stimulated to produce antibodies to continue to do so. The influence of mercury, however, demonstrates that this phenomenon is closely allied to similar actions exhibited by the class of bacteria. If a patient whose serum gives a positive reaction is subjected to mercurial treatment, the reaction becomes negative in several weeks. The mercury has destroyed the stimulant or irritant which has led the cells to the production of antibodies. If this stimulant is excluded, the "lues reagine" disappears from the blood just as bacterial antibodies disappear after the bacteria have been eradicated. Thus there is no basis for attributing to the luetic antibodies any exceptional properties.

Citron's "Biological Mercurial Treatment."

The fact that mercury leads to an alteration in the reaction, prompted the author to employ the *Wassermann test as a guide to the biological mercurial treatment. The aim was not only to cause a disappearance of all manifestations, but to obtain a negative reaction.* It soon appeared that a negative reaction once obtained did not necessarily remain such. As soon as a recurrence set in the reaction became positive again; in fact, the reaction also reappeared without a return of symptoms. In the latter case such a return alone was regarded as a fresh manifestation of a reactivation process and an indication for treatment. It became advisable therefore, to repeat the test at definite intervals and depend upon the return of the reaction for further treatment. This basis of therapy, which at first met with marked opposition, has recently won many followers.

The experiments of Boas in Copenhagen are especially instructive from this point of view.

He examined eighty-two patients with secondary syphilis before and after mercurial therapy. All gave positive reactions before the treatment; after it, seventy-six gave no reaction, six retained the positive reactions; one of the six did not return for observation. Of the remaining five, all had a return of symptoms within one month after cessation of the mercury, while of the seventy-six only three returned with a recurrence. Boas next made observations of sixty-five patients who were in the first three years of their infection, but who gave a negative Wassermann after the treatment. In sixty-two cases, a positive reaction reappeared after one to two months, eight of these having at the same time a recurrence of symptoms; of the remaining fifty-four, nineteen were not treated. They all showed a return of symptoms, but only one and a half months after the appearance of the positive Wassermann. Thus if the scheme of the chronic intermittent mercurial therapy of Neisser and Fournier were followed, these patients would begin to get treatment one and a half months after the active lues had again started, as shown by the positive Wassermann reaction. Of the remaining thirty-five cases all began treatment when the Wassermann test became positive. None of these had any return of symptoms during the following period of observation (three to five months).

The experiments of Boas show distinctly the advantages of the mercurial therapy when based upon the biological reaction instead of upon the schematic, symptomatic, chronic, intermittent treatment of Fournier and Neisser.

At the present day, when the spirochætes can be so readily found in the primary lesion of syphilis, the biological mercurial treatment should be undertaken in the earliest stage. It is possible even to begin at a time when the serum reaction is still negative, but after the spirochæte had been demonstrated. The most ideal cases are those in which treatment is instituted so early that they never develop a positive Wassermann.

Naturally the statement made that mercurial treatment should be continued until the reaction becomes negative may be limited by certain contra-indications in the general condition of the patient which may arise. This must always be considered. Especial difficulty to attain a negative reaction is encountered in those cases where the lues has persisted for many years.

It must be borne in mind that the luetic infection does not always present the typical clinical picture ascribed to it in the text-books. The "Lues asymptomatica," that is, the lues apparently presenting no symptoms, is by no means rare. *To-day one must not wait until the syphilitic patient comes to the physician, but it is the duty of the latter to look for the evidence of syphilis among those related to or associated with infected persons.* If one proceeds in such a systematic method it will be found that the mothers of syphilitic children, so frequently regarded as immune, are in reality not so. In such cases, without any clinical evidence of syphilis, the Wassermann reaction is positive in about 56 to 75 per cent.

This question becomes of utmost importance in the prevention of lues. For example the obligatory examination of the serum of wet nurses has shown that of all such applicants at the Dresden Infant Asylum 10 per cent. gave a positive reaction (Rietschels). On further study it was ascertained

that 75 per cent. of the children of these apparently healthy women gave luetic manifestations immediately or shortly after birth.

Serum Diagnosis of Diseases Caused by Animal Parasites.

In close association with the serum diagnosis of syphilis, complement fixation has been employed as a means for the diagnosis of conditions caused by the animal parasites and especially by the echinococcus. In the serum of patients suffering from these infections, substances are found closely allied to the "lues reagine." They bind complement with an antigen consisting of an extract of the respective worms or hydatid fluid.

Ghedini, Weinberg and Parvu and others have found that in most cases of echinococcus disease, the reaction is positive. If by operation the cyst is only incised the reaction becomes stronger or in few cases appears positive for the first time. After complete excision of the cyst, the reaction disappears. According to Parvu and Laubry, a positive test is found in the spinal fluid only when the echinococcus cysts have invaded the brain.

Ghedini described similar findings, caused by the ascaris, ankylostoma, etc.

CHAPTER XIV.

THE TECHNIQUE OF COMPLEMENT FIXATION.

Original method of Bordet-Gengou. Wassermann-Bruck's modification. Technique of serum diagnosis for syphilis. Echinococcus disease. Differentiation of proteids according to Neisser-Sachs.

I. The Original Method of Bordet-Gengou.

a. The antigen consists of bacteria grown upon agar for twenty-four hours and then suspended in physiological salt solution to make a rather concentrated emulsion.

For typhoid bacteria Bordet and Gengou take 5 c.c. of salt solution to each culture of bacteria.

For tubercle bacilli 80 mg. of the bacteria are suspended in 1 c.c. of salt solution.

b. The serum containing the *antibody* is heated for one-half hour at 56° C. to destroy the complement.

c. As complement, the fresh serum of a normal animal or human being is used.

d. The hemolysin is produced by the inactivated serum of a rabbit that had been immunized against sheep's or goat's erythrocytes, or the serum of a guinea pig injected with rabbit's red blood cells.

e. The respective red blood corpuscles are washed, to free them of their complement containing serum.

A definite amount of bacterial suspension is mixed with varying amounts of inactivated immune serum and a proportional amount of complement is added. These three ingredients are mixed and allowed to remain at room temperature for four to five hours. During this time the complement is fixed if the antigen and antibody are of a homologous nature. In order to see whether this union has taken place or not, hemolysin and erythrocytes are added in a mixture thus prepared: 2 c.c. of inactivated hemolysin + twenty drops of washed blood cells are mixed and allowed to remain together for about fifteen minutes so that the erythrocytes are sensitized, *i.e.*, united with the hemolytic amboceptor. Of this mixture each tube receives 0.1 to 0.2 c.c. If the complement has not become fixed, hemolysis occurs in several minutes. If the complement has become so, hemolysis does not occur; since, however, the hemolysin also contains hemagglutinin, the erythrocytes are agglutinated and sink to the bottom of the tubes.

As control tests, Bordet and Gengou considered the following very necessary:

1. Bacterial suspension + inactivated normal serum (instead of immune serum) + complement (five hours) + hemolysin + blood. Hemolysis must occur, as the normal serum does not contain enough amboceptors to unite with the bacterial suspension and consequently complement remains unbound.

2. Inactivated immune serum + complement (five hours) + hemolysin + blood. Hemolysis results.

3. Inactivated normal serum + complement (five hours) + hemolysin + blood. Hemolysis.

4. Antigen + inactivated immune serum + hemolysin + blood. No hemolysis, as complement is absent.

5. Antigen + inactivated normal serum (five hours) + hemolysin + blood. No hemolysis, as complement is absent.

The following is the chart of the first complement fixation test as originally performed by Bordet and Gengou in 1901 in which pest antibodies were demonstrated in the serum of an immunized horse.

	Antigen.	Antibodies.	Complement.	Hemolysin and erythrocytes.	Result.
1	0.4 pest bacilli emulsion.	1.2 inactive pest serum (horse).	0.2 guinea-pig's serum.	2 drops of rabbit's blood sensitized.	0
2	0.4 pest bacilli emulsion.	1.2 inactive normal serum (horse).	0.2 guinea-pig's serum.	2 drops of rabbit's blood sensitized.	Complete hemolysis.
3	.....	1.2 inactive pest serum (horse).	0.2 guinea-pig's serum.	2 drops of rabbit's blood sensitized.	Complete hemolysis.
4	.....	1.2 inactive normal serum (horse).	0.2 guinea-pig's serum.	2 drops of rabbit's blood sensitized.	Complete hemolysis.
5	0.4 pest bacilli emulsion.	1.2 inactive pest (horse).	.....	2 drops of rabbit's blood sensitized.	0
6	0.4 pest bacilli emulsion.	1.2 inactive normal serum (horse).	.....	2 drops of rabbit's blood sensitized.	0

Employing this method, Bordet and Gengou found positive results with the following combinations:

1. Pest bacilli + pest horse's serum + guinea-pig complement + guinea pig hemolysin + rabbit's blood.

2. Anthrax vaccine + guinea-pig immune serum + guinea-pig complement + guinea-pig hemolysin + rabbit's blood.

3. Typhoid bacilli + guinea-pig immune serum + guinea-pig complement + guinea-pig hemolysin + rabbit's blood.

4. Coli bacilli + guinea-pig immune serum + guinea pig complement + guinea-pig *hemolysin + rabbit's* blood.

5. Typhoid bacilli + human convalescent serum + human complement + guinea-pig hemolysin + rabbit's blood.

6. Killed tubercle bacilli + guinea-pig immune serum + guinea-pig complement + rabbit's hemolysin + goat's blood or sheep's blood.

7. Whooping cough bacilli + patient's serum + guinea-pig's complement + rabbit hemolysin + goat's or sheep's blood.

8. Meningococci + human convalescent's serum + human complement + guinea-pig's hemolysin + rabbit's blood (Cohen).

In addition Widal and Lesourd on examination of sixty-one typhoid cases found fifty-eight with a positive reaction. Foix and Mallein examined twelve cases of scarlet and obtained a positive result in ten cases when the streptococcus grown from a scarlet angina was used as antigen. Antibodies were found on the fourth day. These results were confirmed by Schleissner.

II. Wassermann-Bruck's Modification.

a. Antigen.—Instead of entire bacteria, only bacterial extracts are employed. These are made in the same manner as the artificial aggressins.

For typhoid bacteria Leuchs advises that the bacterial suspension should first be killed for twenty-four hours at 60° C. and then shaken for two days. In tuberculosis good results are obtained by using Koch's preparation of old and new tuberculin.

The bacterial extracts when very fresh contain a great deal of precipitinogen which diminishes in several days and finally disappears. Its presence does not disturb complement fixation. *The bacterial extracts must be well protected from light and kept in the cold.*

After the extract has stood for some time a sediment forms; under no circumstance should this be disturbed or shaken. The required amount of antigen should be carefully poured off, and not pipetted off. Just as soon as the required amount is obtained, the extract should be returned to the ice-box.

b. The antiserum is inactivated by heating, even if the serum is old and contains very little or no complement.

Old, non-heated serum is often antihemolytic. Temperatures over 60° C. should be strictly guarded against as the amboceptors may be destroyed. Heating for a period longer than one-half hour may make a serum anticomplementary, *i.e.*, bind complement. Sera containing bile at times prevent hemolysis. Chylous sera obtained during the period of digestion and milky sera seen in nursing women do not differ from the normal.

Exudates, transudates, and spinal fluids are treated like sera.

c. Complement is obtained by killing a guinea-pig and using its serum while fresh. The serum preserved in "Frigo" is, according to Sterns, not reliable.

d. Hemolysin is represented by the inactivated serum of a rabbit that has been immunized against sheep's red blood cells.

e. The twice washed sheep's red blood cells are used as erythrocytes.

These five substances are placed into the test tubes in the following order: antigen, inactivated antiserum, complement; they are thoroughly mixed by shaking and placed into the incubator for one hour in order to hasten their union. After this interval the inactivated hemolysin and the red blood cells are added as indicator. The mixtures are again returned to the incubator to promote hemolysis. Like in all biological reactions, the quantitative relationship of these various ingredients determines to a great extent the final result of the complement fixation test. As for antigen and antibody the experiments of Weil and Nakayama must be considered; these are to the effect that only one-half of the maximum non-hemolytic dose of each ingredient is employed. With this point in view, preliminary tests determining the proper dosage of each must be performed.

The amount of complement used is always constant. In Wassermann's laboratory 1 c.c. of the dilution 1:10 represents the quantity chosen. Of the hemolysin the two fold or three fold titre dose is taken and of the erythrocytes 1 c.c. of a 5 per cent. suspension in normal saline solution suffices. Each of the five elements is diluted with saline to make up 1 c.c. so that at the completion of the test all the tubes contain 5 c.c. Quite a difference arises if an individual test is performed with a constant quantity of serum and diminishing doses of bacterial extract or reversely. Important tests should be carried out by both methods. The necessary controls are:

1. The double dose of antigen + complement + hemolysin + blood, to prove that the dose employed in the test is correct (Weil and Nakayama).

2. The double quantity of serum + complement + hemolysin + blood, to prove that the dose of serum employed is correct (Weil and Nakayama).

3. The "system control"; blood + complement + one-half amount of hemolysin, to show that the test was performed with double the hemolytic dose.

4. Blood + salt solution, to prove that the salt solution is isotonic.

In addition, it is advisable to repeat the test with inactivated normal serum substituted for the immune serum and another with a foreign instead of a homologous antigen.

These controls assure beyond doubt the specificity of the reaction.

The accompanying chart represents schematically all that has been discussed.

Titration of a Meningococcus Serum Obtained from the Horse.

a. Diminishing Quantities of Antigen.

Antigen.	Antibodies.	Complement.	Hemolysin.	Erythrocytes.	Results.
0.25 c.c. Meningococcus extract	0.1 c.c. inactive immune serum.	0.1 c.c. guinea-pig's serum.	0.002 c.c. inactive rabbit's (sheep) serum.	1 c.c. 5% sheep's blood	0
0.1 c.c.	"	"	"	"	0
0.05 c.c.	"	"	"	"	0
0.01 c.c.	"	"	"	"	0
0.005 c.c.	"	"	"	"	Incomplete
0.001 c.c.	"	"	"	"	Incomplete
0.0005 c.c.	"	"	"	"	Complete
1.0 c.c.	.....	"	"	"	Incomplete
0.5 c.c.	.....	"	"	"	Complete
0.25 c.c.	.....	"	"	"	Complete

Antigen.	Antibodies.	Complement.	Hemolysin.	Erythrocytes.	Results.
.....	Inactive immune serum.				
	0.4 c.c.	"	"	"	Incomplete
.....	0.2 c.c.	"	"	"	Complete
.....	0.1 c.c.	"	"	"	Complete
.....	.....	"	0.001 c.c.	"	Complete
.....	.....	.....	.....	"	0
Meningococcus extract.	Inactive normal horse's serum.	"	0.002 c.c.	"	Almost Complete
0.25 c.c.	0.1 c.c.				
0.1 c.c.	0.1 c.c.	"	"	"	Complete
.....	0.2 c.c.	"	"	"	Complete
Staphylococcus extract.	Inactive meningococcus serum.	"	"	"	Complete
0.25 c.c.	0.1 c.c.				
0.1 c.c.	0.1 c.c.	"	"	"	Complete
0.5 c.c.	.....	"	"	"	Complete

The titre of the meningococcus serum is 0.001 c.c. of antigen. Since 1.0 c.c. of antigen binds 0.1 complement and 0.5 c.c. does not interfere with hemolysis, the maximum dose of antigen which may be used for the trial is 0.25 c.c.

Inasmuch as 0.4 c.c. of the inactivated serum binds a part of the complement, and 0.2 does not at all interfere with hemolysis, the maximum dose of serum to be employed is 0.1 c.c.

The positive reaction must be attributed to the interaction between antigen and antibody, as hardly any complement fixation takes place by using inactivated normal serum with 0.25 c.c. of antigen. That the reaction is specific is shown by hemolysis occurring when the homologous antigen is substituted by a staphylococcus extract.

b. Same with Diminishing Amounts of Serum.

Antigen.	Antibodies.	Complement.	Hemolysin.	Erythrocytes.	Result.
0.25 c.c. meningococcus extract	0.1 c.c. inactive meningococcus serum.	0.1 c.c. guinea-pig's serum.	0.002	1 c.c. 5% sheep's blood	0
"	0.05 c.c.	"	0.002	"	0
"	0.01 c.c.	"	0.002	"	0
"	0.005 c.c.	"	0.002	"	0
"	0.001 c.c.	"	0.002	"	Almost 0
"	0.0005 c.c.	"	0.002	"	Incomplete.
"	0.0001 c.c.	"	0.002	"	Complete.
0.5 c.c. meningococcus extract	.....	"	0.002	"	Complete.

Antigen.	Antibodies.	Complement.	Hemolysin.	Erythrocytes.	Results.
.....	0.2 c.c.	"	0.002	"	Complete.
.....	.....	"	0.001	"	Complete.
.....	.....	.....	.....	"	0
0.25 c.c. meningococcus extract.	Inactive normal horse's serum. 0.1 c.c.	"	0.002	"	Almost complete.
"	0.05 c.c.	"	0.002	"	Complete.
.....	0.2 c.c.	"	0.002	"	Complete.
0.25 c.c. staphylococcus extract.	0.1 c.c. inactive meningococcus serum.	"	0.002	"	Complete.
0.5 c.c. staphylococcus extract.	.....	"	0.002	"	Complete

With 0.25 c.c. of antigen the titre of this meningococcus serum is 0.0005 c.c. of serum. One could with this constant quantity of serum, and varying quantities of antigen, titrate the minimum amount of antigen necessary for complement fixation. It would even be preferable for such a test to employ 0.005 c.c. of the serum, as this amount surely binds no complement. If such a titration is undertaken it will be found that 0.005 c.c. of serum with 0.05 c.c. of extract can bind 0.1 c.c. of complement.

Similarly the antibodies contained in the blood serum or spinal fluid of a patient can be determined by means of complement fixation.

If it is desired to demonstrate the antigen instead of antibody, one proceeds as follows:

c. Demonstration of meningococcus antigen in the spinal fluid of a patient with a possible meningitis.

Antigen.	Antibodies.	Complement.	Hemolysin.	Blood.	Results.
0.5 c.c. active spinal fluid from patient.	0.1 c.c. inactive horse's meningococcus serum.	0.1	0.002	1 c.c. 5%	0
0.3 c.c.	0.1 c.c.	0.1	0.002	1 c.c. 5%	Incomplete.
0.1 c.c.	0.1 c.c.	0.1	0.002	1 c.c. 5%	Complete.
.....	0.2 c.c.	0.1	0.002	1 c.c. 5%	Complete.
1.0 c.c.	.....	0.1	0.002	1 c.c. 5%	Almost complete.
0.6 c.c.	.....	0.1	0.002	1 c.c. 5%	Complete.

Antigen.	Antibodies.	Complement.	Hemolysin.	Erythrocytes.	Results.
.....	.....	0.1	0.001	1 c.c. 5%	Complete.
.....	.....	.....	.....	1 c.c. 5%	0
0.5 c.c. active normal spinal fluid.	0.1 c.c.	0.1	0.002	1 c.c. 5%	Complete.
1.0 c.c.	.....	0.1	0.002	1 c.c. 5%	Complete.
0.5 c.c. of active spinal fluid from patient.	0.1 c.c. inactive normal horse's serum.	0.1	0.002	1 c.c. 5%	Almost complete.
0.3 c.c.	"	0.1	0.002	1 c.c. 5%	Complete.
0.05 c.c. meningococcus extract.	0.005 c.c. inactive meningococcus serum.	0.1	0.002	1 c.c. 5%	0 Look at previous test.

The spinal fluid contains meningococcus antigen thus proving that the patient is suffering from epidemic cerebrospinal meningitis. Neither the double amount of serum, a double amount of antigen, a mixture of normal spinal fluid with specific serum, nor normal serum with the specific spinal fluid binds complement. Only a mixture of meningococcus extract and specific serum gives complement fixation.

The results gotten by complement fixation greatly depend upon the quantitative relationship of the various ingredients. The affinity toward the complement existing between the antigen and amboceptor on the one hand, is balanced by that between the hemolysin + blood on the other. By modifying their quantitative proportions different results may be obtained. If for example the strength of the hemolysin is excessively increased, it is possible that the previously bound complement is again detached and hemolysis ensues. Originally the results were read after the mixtures had remained two hours in the incubator and twenty-four hours in the ice-box. At the present, most authorities agree to read the results at a time when the control tubes are ready; that is when the complement is bound or hemolysis has been completed in those tubes in which these respective phenomena should occur.

Wassermann's modification of the Bordet-Gengou method was first practically employed for the titration of the therapeutic meningococcus serum. One cannot, however, correctly judge the prophylactic or curative value of a serum by its antibody content as they do not run hand in hand [R. Kraus, Garbat, Citron.] The complement fixation method was also applied by Bruck for the diagnosis of epidemic meningitis; by Müller and Oppenheim for the diagnosis of general gonococcus infections (gonorrheal arthritis, iridocyclitis, etc.) and by Hirschfeld, Leuchs and Schöne and others, in typhoid—all with favorable results.

III. Serum Diagnosis of Syphilis.

a. Wassermann's Technique.

The technique of this reaction as carried out in Wassermann's laboratory is practically identical with that just described for the diagnosis of bacterial infections. The preparation of the antigen varies slightly.

The liver obtained from a syphilitic fetus is weighed and cut up into fine pieces. Four times its weight of 1/2 per cent. of carbolic solution in saline is added and the mixture placed into a brown bottle and shaken for twenty-four hours. It is then centrifu galized until the larger liver remnants settle to the bottom and a somewhat turbid fluid remains above. The latter is poured off into a brown bottle and placed into the ice-box. After several days of sedimentation, the fluid assumes a yellowish-brown opalescence and can now be used as a luetic antigen. It should not be exposed to light and heat, should not be shaken, and its contents should not be pipetted off, but carefully poured off without disturbance to the sediment.

By titration of the extract, that dose is determined which does not of itself bind complement. Only such extracts are kept which in the dose of 0.4 c.c. do not interfere with hemolysis.

Control tests should also be made to ascertain whether the organ extract has any tendency of its own to hemolyse red blood cells without the presence of complement or hemolysin.

Not every luetic extract can serve as antigen for complement fixation. During the process of extraction a number of other substances, both normal and pathological, may be drawn from the luetic liver besides that agent necessary for the Wassermann test. These undesired ingredients may interfere with the efficiency of the extract. For this reason a great number of known positive and negative sera should be tested with each new extract, and only if the results are absolutely correct should it be employed as antigen.

In the early work of Wassermann the antigen was described as deteriorating very easily; its activity would either be entirely destroyed or it would become anticomplementary. The author is firmly convinced that these changes are brought about by careless handling of the extract or its exposure to light. If properly taken care of, its activity remains constant.

From practical experience, it has been found that extracts which must be used in amounts less than 0.1 c.c. are as a general rule unsatisfactory. Similarly, the luetic sera are most active when doses of 0.2 and 0.1 c.c. are employed. Amounts greater than 0.2 may result in an unspecific reaction. The most favorable combinations are,

0.2 c.c. of extract + 0.2 c.c. serum.
0.1 c.c. of extract + 0.1 c.c. serum.

The accompanying table presents the titration of an antigen in detail.

a. Preliminary Test—Titration of the Antigen.

Antigen.	Complement.	Hemolysin.	Blood.	Result.
0.8 c.c. luetic extract	0.1	Twice the hemolytic dose.	1 c.c. 5%	Incomplete hemolysis.
0.6 c.c. luetic extract	0.1	Twice the hemolytic dose.	1 c.c. 5%	0
0.4 c.c. luetic extract	0.1	Twice the hemolytic dose.	1 c.c. 5%	Complete hemolysis.
0.2 c.c. luetic extract	0.1	Twice the hemolytic dose.	1 c.c. 5%	Complete hemolysis.
0.8 c.c. luetic extract	.....	.....	1 c.c. 5%	Incomplete.
0.6 c.c. luetic extract	.....	.....	1 c c. 5%	0
0.4 c.c. luetic extract	.....	.....	1 c.c. 5%	0
0.2 c.c. luetic extract	.....	.....	1 c.c. 5%	0

The test proves that 0.4 c.c. of extract is not able to bind 0.1 c.c. of complement. That 0.8 c.c. of lues extract causes only an incomplete hemolysis, while 0.6 c.c. produces no hemolysis whatever, is explained not by its lessened tendency of binding complement, but by the greater amount of hemotoxin which 0.8 c.c. possesses.

b. Examination and titration of 4 luetic sera by Citron's method (*see plate* II.)

The technical details of the test are as follows:

Three est tubes are assigned for each test and placed into a test tube rack. The name of the patient is written upon the first of these tubes. Another rack contains one tube for each patient and labelled accordingly. In addition there is an "antigen tube," which is placed into the first rack at the end of all the other tubes; also a "normal extract," a "system," "complement," and "blood control tube," which are placed into the second rack. The amount of syphilitic antigen required for the entire work is calculated as follows. For each test 0.3 of antigen is required; for five cases (including controls) 1.5

Luetic extract.	Serum.	Complement.	Hemolysin; 1 c.c.	Sheep's blood.	Result of hemolysis.	
1. 0.2	0.2 Ser. I. Tabes untreated; without luetic history.	0.1	1 : 1000	1 c.c. 5%	No hemolysis.	++++
2. 0.1	0.1 As above.	0.1	1 : 1000	1 c.c. 5%	No hemolysis.	
3. ...	0.2 * As above.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
4. 0.2	0.2 Ser II. Secondary lues untreated.	0.1	1 : 1000	1 c.c. 5%	No hemolysis.	+++
5. 0.1	0.1 As above.	0.1	1 : 1000	1 c.c. 5%	Incomplete hemolysis.	
6. ...	0.2[1] As above.	0.1	1 : 1000	1 c c. 5%	Complete hemolysis.	

Luetic extract.	Serum.	Complement.	Hemolysin; 1 c.c.	Sheep's blood.	Result of hemolysis.	
7. 0.2	0.2 Ser. III Tabes. Many inunction courses.	0.1	1 : 1000	1 c.c. 5%	No hemolysis.	++
8. 0.1	0.1 As above.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
9. ...	0.2 As above.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
10. 0.2	0.2 Ser. IV Gallstones. Lues seventeen years ago. Much treatment. No symptoms for ten years.	0.1	1 : 1000	1 c.c. 5%	Trace of binding; almost but not quite complete hemolysis	±
11. 0.1	0.1 As above.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
12. ...	0.2 * As above.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
13. 0.2	0.2 Negative control serum (carcinoma hepatis).	0.1	1 :1000	1 c.c. 5%	Complete hemolysis.	—
14. ...	0.2[1] As above.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
15. 0.1	0.1 Strongly positive control serum. (Lues maligna).	0.1	1 : 1000	1 c.c. 5%	No hemolysis.	++++
16. ...	0.2 As above.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
17. 0.2	0.2 Weakly positive control serum. Primary lesion.	0.1	1 : 1000	1 c.c. 5%	Incomplete hemolysis.	+
18.	0.2[1] Weakly positive control serum. Primary lesion.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
19. 0.4	.....	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
Normal extract.						
20. 0.2	0.2 Serum I.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
21. 0.2	0.2 Serum II.	0.1	1 : 1000	1 c.c. 5%	Incomplete hemolysis.	
22. 0.2	0.2 Serum III.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
23. 0.2	0.2 Serum IV.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
24. 0.2	0.2 Negative control serum.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
25. 0.2	0.2 Strongly positive control serum.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
26. 0.2	0.2 Weakly positive control serum.	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
27. 0.4	.....	0.1	1 : 1000	1 c.c. 5%	Complete hemolysis.	
28. ...	.....	0.1	1 :2000	1 c.c. 5%	Complete hemolysis.	
29. ...	.....	0.1	.....	1 c.c. 5%	No hemolysis.	
30. ...	.....	.....	.....	1 c.c. 5%	No hemolysis.	

c.c. are needed +0.4 c.c. for the antigen control tube = 1.9 c.c. in all or 2.0 c.c. in round numbers. This amount is diluted with normal salt solution in the proportion of 1:5 so that 8 c.c. of saline are added (=2:10), 1 c.c. of this dilution contains 0.2 antigen and 1/2 c.c. contains 0.1 antigen. The first tube (1, 4, 7, 10, etc., in diagram) of every test therefore receives 1 c.c., the second tube 1/2 c.c., the third tube nothing, the antigen tube (tube 19) 2 c.c. Physiological salt solution is added to make up 1 c.c. in each tube; first tube nothing; second tube 1/2 c.c.; third tube 1 c.c. of saline.

The normal extract required for the tubes in the second rack is similarly estimated, 0.2 c.c. is needed for each test, 5 (tests) × 0.2 = 1.0 + 0.4 for the antigen control tubes = 1.4 or 1.5 c.c. in round numbers. For purposes of dilution 1 : 5, 6 c.c. of salt solution are added and 1 c.c. (=0.2 of extract) placed into each of the tubes (20 to 26) and 2 c.c. into the normal extract control test-tube (tube 27). In this series also, salt solution is added to make up equal quantities of 1 c.c.: first tube nothing, second tube 1/2 c.c., third tube 1 c.c.; antigen tube 27, nothing; system (28), complement (29) and blood (30) control tubes each 1 c.c.

The second ingredient of the test is next added, *i.e.*, the respective serum. This is not diluted but added directly; 0.2 c.c. into the first tube of each test; 0.1 c.c. into the second; 0.2 c.c. into the third tube; also 0.2 into the control series of tubes labelled with the patient's names in the second rack. Salt solution is again added to make up to the equal quantity of 2 c.c. in each tube, thus: 0.8 c.c. into first, 0.9 c.c. into the second, 0.8 c.c. into the third tube, and 0.8 c.c. into the control series; nothing into the antigen tubes, 1 c.c. into system, complement, and blood control tubes.

The addition of complement follows next. Each tube, except the blood tube, receives 0.1 of complement. Thus the tubes are counted and if, for example, ninteen tubes are present 19 × 0.1 c.c. complement is taken, or in round numbers 2 c.c.

Complement is always diluted 1 : 10, or 2 c.c. complement + 18 c.c. saline, so that each tube except the blood tube (30) receives 1 c.c. of this diluted complement Tube (30) receives 1 c.c. of saline instead. All tubes are then carefully shaken and the racks placed into the incubator for one hour.

During this time, the hemolysin and washed red blood cells are properly diluted. The red blood cells are made up in a 5 per cent. suspension of which each tube will receive 1 c.c. Thus in the present test thirty tubes exist, requiring 30 c.c. of blood suspension; since 1 c.c. of washed blood when diluted 1: 20 will supply twenty tubes, for 30 c.c. about 1 1/2 c.c. of blood will be required, or 2 c.c. will make 40 c.c. of a 5 per cent. blood suspension.

As for the *hemolysin*, its titer for example is 1 : 2000 and it is employed in the dilution of 1 : 1000. Each tube except the blood and complement tubes will receive 1 c.c. of the diluted hemolysin; 1 c.c. of the latter if diluted properly would give 1000 c.c.; 0.1 of the hemolysin which is the smallest amount that can be measured out, will give 100 c.c. Every tube except 28 to 30 will receive 1 c.c. of the hemolysin dilution 1 : 1000. Tubes 29 and 30 will receive none (replaced by saline), tube 28 will receive 1/2 c.c. of this hemolysin and 1/2 c.c. of saline.

After an hour's incubation, each tube receives 1 c.c. of R. B. C. and 1 c.c. of the hemolysin just mentioned. If it is desired to hasten the results, it is advisable to mix a sufficient equal quantity of R. B. C. and hemolysin solution (30 c.c. of each) and allow the mixture to remain in the incubator for a short time before the hour's incubation

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[1] 0.4 c.c. of luetic serum frequently binds complement of its own accord. Experience has shown that if 0.2 c.c. does not bind complement and 0.2 c.c. of serum + 0.2 c.c. of antigen does bind complement, the unknown serum is surely of luetic origin.

is up. Then instead of adding 1 c.c. of these ingredients separately, 2 c.c. of the mixture is added to all except tubes 28 to 30. Tubes 29 and 30 receive 1 c.c. of blood and 1 c.c. of saline and tube 28 1 c.c. of blood, 1/2 c.c. of hemolysin and 1/2 c.c. of saline which have been sensitized.

The various strengths of the resulting reactions are differentiated as follows:

a. Tubes 1 and 2 show complete absence of hemolysis: ++++ } Strongly positive.

b. Tube 1 shows complete absence of hemolysis and 2 shows faint hemolysis: +++ } Strongly positive.

c. Tube 1 shows complete absence of hemolysis and 2 shows complete hemolysis: ++ } Weakly positive.

d. Tube 1 shows partial hemolysis and 2 shows complete hemolysis: + } Weakly positive.

e. Tube 1 shows doubtful binding and 2 shows complete hemolysis: ± } Doubtful.

f. Tubes 1 and 2 show complete hemolysis: −, Negative.

When a series of tests is to be performed, it is advisable to include in the reaction three already tested sera, one strongly positive, another weakly positive and a third, negative, so that the new result can be more readily compared. In this way absolutely reliable and constant values will be obtained.

Every new antigen should be tested for four weeks before its practical value can be assured. During this month, all the tests should be done with both the old and new extract and only if their results are equal should the new extract be employed. The author is in the habit of mixing the new antigen with the old one after the former has proved itself efficient. Occasionally the new antigen varies in strength from the old one. In such a case, if stronger, it must be used in a smaller dose (0.18 and 0.9) or if weaker, must be used in larger dose (0.22 and 0.11). Shaking up of the antigen should be strictly guarded against.

In order to control the effect of normal liver substances contained in the antigen, an extract is prepared in an analogous method from normal fetal liver (normal antigen).

A strongly positive Wassermann reaction indicates the presence of a luetic infection. A weakly positive result can be similarly interpreted if the serum control tube (Tube No. 3) is completely hemolysed. If, however, the latter still shows some non-hemolysed red blood cells, the + reaction must be considered as ± or a reaction of indefinite nature. Only exceptionally are such doubtful reactions found in perfectly healthy individuals, although they are more often encountered in different infectious diseases (typhoid, measles, scarlet) and tumors. A positive diagnosis of lues should never be made upon a ± reaction. On the other hand if there is a history of lues, or clinical evidences of the same, a ± reaction is to be interpreted as + and *should* warrant further specific therapy. As an end result of specific therapy

a± reaction is not sufficient. Not before an absolutely negative reaction has been attained should specific therapy cease.

Several authorities consider only such tests as positive where there is complete absence of hemolysis. This principle is proven as incorrect by their own statistics; a great number of their surely syphilitic cases give a negative reaction.

If the third tube (serum control) does not hemolyse, the test can neither be considered as positive nor negative. Very frequently the third tube of very strongly positive cases will hemolyse very much more slowly than negative cases; these tests must therefore remain in the incubator for a longer period than the negative or weakly positive ones [ed]. and until the serum tube is completely hemolysed.

b. Modifications of Wassermann's Technique.

On account of the somewhat complex technique of the reactions, numerous attempts have been made to simplify the test in one way or another. The greatest difficulty lay in the preparation of a suitable antigen. From the sundry modifications and improvements made in this respect, perhaps the most important was announced simultaneously by Landsteiner, Müller and Pötzl, and Porges and Meier.

They showed that by alcoholic extraction of luetic and even normal organs of human beings and lower animals, substances were obtained which could be used as a substitute for the aqueous syphilitic antigen. The belief therefore arose that the active agents in the luetic extract belong to the class of lipoids, and Porges and Meier endeavored to isolate them from the serum. Thereupon it became evident that lecithin could replace the antigen, but only up to a certain point. Further study by H. Sachs lead to the adoption of entire formulæ for artificial antigens.

The new principle disclosed by these discoveries lead to many modifications in the preparation of the antigen, the main advantage of which consisted in bringing the reaction into more general use and application. The previous necessity of making an extract from the liver of a luetic fetus somewhat limited this. The Wassermann reaction became in a short period of time much more popular, *although one could not adhere to it with the same idea of specificity as before.*

Other changes in the reaction, referred to the serum for examination. H. Sachs demonstrated that the inactivation at 56° C. destroyed a great part of luetic the "reagine." The dispensation of the latter was therefore recommended. It soon became evident, however, that by so doing, a great number of normal and non-luetic pathological sera gave a positive reaction. *It is best therefore that this modification should by all means be discarded.*

As all fresh sera contain complement, the addition of guinea-pig's complement seemed superfluous if the serum for examination is employed in an active form. The following combination was therefore proposed:

1. Luetic extract or one of its substitutes.

2. Active luetic serum (contains luetic "reagine" + complement). One hour in incubator.

3. Inactive hemolysin.
4. Red blood cells.

In view of the above-mentioned objections, to wit, the too frequent positive results, this modification although advised by divers authorities, Stern and others, should not be employed.

Not only the addition of complement, but also of immune hemolysin can be discarded, because every serum normally contains hemolytic antibodies for foreign species of blood. The contraindication for the transfusion of foreign blood depends upon this principle.

Accordingly, some authors advise the following schemes:

1. Luetic extract or its substitute.
2. Inactive luetic serum ("luesreagine" + hemolysin).
3. Complement. One hour in incubator.
4. Washed erythrocytes of sheep.

1. Luetic extract or its substitute.
2. Active luetic serum (luesreagine + complement + normal hemolysin), one hour in incubator.
3. Washed erythrocytes of sheep.

The advantage of these modifications is supposed to exist in the omission of the immune hemolysin. The preparation and preservation of this ingredient is, however, technically so simple that this advantage is only theoretical. Bauer believes that this change is preferable to the classical method for the reason that with the latter, the x amount of normal hemolysin is always added to the constant amount of immune hemolysin, thereby resulting in a different quantity of the same in each test. Experience has, however, shown that the faint trace of normal hemolysin never influences the result of the test. At times so little normal hemolysin will exist in a patient's serum that it becomes necessary to add some serum of another normal patient. Such manipulations lead to new difficulties so that taken all in all, this innovation offers no advantages and should therefore not be accepted.

Brieger and Renz have recently advised the substitution of potassium chlorate for the immune hemolysin. Had this been correct the biological bases of the Wassermann reaction would have been undermined. Garbat and Munk have, however, shown that in this modification $KClO_3$ is entirely inert and that the reaction depends upon the normal hemolysin in human serum against sheep's erythrocytes.

Several workers in this field believed that it would be advantageous to use a different species of blood in place of sheep's erythrocytes.

The only suggestion which sounds theoretically correct is that of Noguchi, who employs human erythrocytes and the serum of a rabbit immunized against human red blood cells. In this way he attempts to exclude the x normal hemolysins, as human serum possesses no hemolysins against human blood cells.

1. Syphilis extract or its substitute.
2. Inactive syphilis-serum.
3. Complement from human being or guinea-pig, one hour in incubator.

(2 and 3: An active syphilis serum.)

4. Immune hemolysin of a rabbit against human erythrocytes.
5. Washed human erythrocytes.

1. Syphilis extract.
2. Active defibrinated syphilitic blood. (Erythrocytes, "reagine," complement), one hour in incubator.
3. Immune hemolysin of rabbit (injected with human blood).

From a practical standpoint, however, no distinct advantage is offered by these modifications. In fact, it is the claim of Wassermann and his pupils that by the use of human blood, the error tends towards the opposite direction, *i.e.*, the percentage of positive results obtained are higher than is actually the case.

The number of modifications have become so numerous that almost every one employs his own "method." *There is absolutely no necessity for this*, as an innovation justifies its existence only if it is a distinct improvement, *i.e.*, discloses a new fact or radically simplifies the old.

It is the classical Wassermann reaction performed in the original manner which has taught physicians how valuable a clinical aid it is. Their knowledge has not advanced a step further even with all the new changes. A single advantage only has been instituted through all this agitation, and that was the discovery that the luetic antigen can be replaced by *the alcoholic extract of guinea-pig's heart. In important differential diagnosis, however, even this kind of extract should not be considered as specific as luetic liver antigen.*

For general work, however, its employment may be of service.

The antigen of Landsteiner, Müller and Pötzl is prepared as follows:

The heart of a guinea-pig is washed free of blood, its muscular part finely divided or macerated in a mortar and then extracted with 95 per cent. of alcohol for several hours at 60° C. One gram of the heart substance should be mixed with 5 c.c. of the alcohol. The material is then passed through filter-paper, the filtrate being kept at room temperature. [The editor prepares the alcoholic extract by simply placing the finely divided guinea-pigs' hearts into 95 per cent. alcohol and allowing them to remain there for almost four weeks for purposes of extraction. At the end of this period the alcoholic solution is titrated and can be employed as antigen.]

Drop Method.

These authors also employ the so-called drop method:

Ten drops of saline and 1 drop of normal guinea-pig's serum as complement is placed into each test-tube. The individual tubes then receive the following ingredients:

First tube: One drop of the inactivated serum for examination.
Second tube: Same as one + 2 drops of the alcoholic heart extract.
Third tube: One drop of inactivated, surely luetic serum.
Fourth tube: Same as three + 2 drops of alcoholic heart extract.
Fifth tube: One drop of inactive normal serum.
Sixth tube: Same as five + 2 drops of alcoholic heart extract.
Seventh tube: Two drops of extract.

The tubes are well shaken and placed into the incubator for one hour at 37° C. Then 1 drop of a 50 per cent. (!) suspension of washed sheep's erythrocytes and 1 drop of hemolysin (double the maximum hemolytic titer) are added. After one-half hour in the incubator, the results are read off.

Bauer's Modification.

Bauer entirely excludes the immune hemolysin. His reaction requires the following ingredients:

1. Fresh guinea-pig's complement.
2. Alcoholic organ extract.

3. Five per cent. sheep's red blood corpuscles.

4. and 5. The inactivated serum for examination and an inactive normal control serum.

Four tubes are required for the reaction:

First tube: 0.2 serum, 1.0 c.c. organ extract in dilution 1 : 5 and 1 c.c. complement 1 : 10.

Second tube: Same as 1, but instead of organ extract, 0.85 per cent. sodium chloride.

Third tube: Two-tenths normal serum, organ extract and complement as in tube 1.

Fourth tube: Same as third tube, but instead of organ extract 0.85 per cent, saline.

The tubes are placed into incubator for one-half hour and then 1 c.c. of a 5 per cent. red blood cell emulsion is added.

After fifteen to forty-five minutes tubes 2, 3, and 4 show hemolysis, while tube 1 shows hemolysis or not, depending upon the absence or presence of syphilis.

Lipemic serum is not suitable for the reaction.

Bauer asserts that this method gives results identical with those obtained by the Wassermann tests. Heinrichs, Bering and others confirm Bauer's findings.

If the alcoholic extract made from luetic or normal human or animal organs is diluted with physiological saline, a milky opalescent solution results. The grade of turbidity of the resulting solution depends upon the rapidity with which the saline for dilution is added. If the first 15 to 20 drops of the latter are added slowly, the resulting solution will be much more turbid than if the saline is added quickly. Sachs first observed this phenomenon and stated that the stronger the turbidity the more active is the power of the antigen to bind complement.

The editor has worked with the guinea-pig's heart extract in several thousand tests and has found it giving perfect results. The amount usually used is 0.2 to 0.1 c.c. in the first test-tube and 0.1 to 0.05 in the second test-tube as determined by titration. When the antigen is diluted (either 1:5 or 1:10) the first c.c. of saline should be added drop by drop and shaken, thus producing a distinctly opalescent solution.

The author refrains from describing any other modifications in detail as they have not been verified sufficiently to merit a position in this important field of serum diagnosis. This holds true especially for the recently advised quick and easy short cuts by the use of the various ingredients dried on paper. In order, however, that one may acquaint himself with these modifications, if he so desires, the reference of their original publications are here given.

Tschernogubow, Berlin. Klin. Wochenschr., 1908, No. 47, and Deutsche Med. Wochenschr., 1909, No. 15.

Weidanz, Deutsche Med. Wochenschr., 1908, No. 48, Refer.

Noguchi, Journal of Americ. Medic. Associat., 1908, No. 22, u. Münch. Med. Woch., 1909, No. 10.

Hecht, Wien. Klin. Wochenschr., 1908, No. 50, and 1909, No. 10.

Fleming, Lancet, 1909, 4474.

Stern, Zeitschr. f. Immunitätsforschung, 1909, Bd. I.

Bauer, Deutsche Med. Wochenschr., 1909, No. 10.

IV. Serum Diagnosis of Echinococcus Disease.

The technique of this reaction is practically the same as described for the Wassermann test.

As antigen the cystic fluid of the human being or sheep is employed. The latter according to Weinberg is preferable, as human hydatid fluid sometimes reacts with normal serum.

The following is Weinberg's outline for performing the test:

Hydatid fluid from sheep.	Inactive serum from patient.	Complement.	Hemolysin.	Blood.	Results.
0.4	0.5	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	0
0.4	0.4	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	0
0.4	0.3	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	Incomplete.
0.4	0.2	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	Complete.
0.4	.....	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	Complete.
.....	0.5	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	Complete.
.....	0.4	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	Complete.
.....	0.3	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	Complete.
.....	0.2	0.1	2×hemolytic dose.	1 c.c. 5% sheep's blood.	Complete.

Bauer's modification as employed for the Wassermann test can also be employed here.

V. The Differentiation of Proteids by the method of Neisser and Sachs.

This technique varies only in a few details from the method advanced later on by Wassermann and Bruck for the diagnosis of bacterial infections.

Neisser and Sachs do not employ a constant amount of complement (0.1), but first titrate the complement against a dose of hemolysin double its hemolytic titer. For the test one and a half to two times the complement titer is necessary. The hemolysin consists of the serum of a rabbit immunized against ox's blood. This hemolysin acts both for ox's and sheep's erythrocytes.

The amount of antiserum (for example antihuman serum) used for the test, is influenced by two factors.

1. An excess of antiserum can interfere with the fixation of complement.
2. The antiserum if used in large quantities can bind complement of its

own accord; without the addition of the human serum, for example. It is therefore best, to ascertain by titration, the smallest quantities of antiserum which may satisfactorily be employed, as the complement fixation test must be sufficiently delicate to determine 0.0001 c.c. of the human serum.

Diminishing amounts of antiserum are mixed with .0001 c.c. of human serum and 0.1 c.c. of complement. A control series is made wherein the human serum is replaced by the same amounts of saline. (The quantity in all tubes should be made uniform by the addition of normal salt solution, but the total amount of fluid in each tube should not exceed 2.3 to 2.5 c.c.). The tubes are incubated for 1 hour and the hemolytic amboceptor and red blood cells added. After two hours at 37° the results are read off. The .0001 c.c. of the serum is added in the form of 0.2 c.c. of a 1 : 2000 dilution.

TABLE III.

Amounts of antiserum in cubic-centimeters.	Series A contains antiserum +0.0001 c.c. human serum (1 : 2000.02) +0.1 guinea-pig's serum. One hour at 37°. +0.001 c.c. of amboceptor +1 c.c. of 5 per cent. ox's-blood.	Series B (control) contains antiserum +0.2 c.c. physiological saline +0.1 of guinea-pig's serum. One hour at 37°. +0.001 c.c. of amboceptor +1 c.c. 5 per cent. of ox's-blood.
	Hemolysis.	Hemolysis.
0.1	Faint trace.	Complete.
0.075	Faint trace.	
0.05	0	
0.035	0	
0.025	0	
0.015	Trace.	
0.01	Slight.	
0	Complete.	

The antiserum itself as seen in the control series (B) does not, even the amount of 0.1 c.c. (larger quantities never come into consideration) exhibit any tendency to interfere with hemolysis. On the other hand, series (A) shows that the larger amounts of the antiserum do not bind complement as thoroughly as the medium doses. The zone of complete complement fixation lies between 0.05 and 0.025 c.c. of the antiserum. It is advisable as a general rule to choose about one and one-half to two times this minimum quantity. Thus from Table III it can be noted that 0.2 c.c. of a 1 : 6 dilution would be well adopted as a test dose for complement fixation. If it is required to know how delicate the complement fixation reaction can be with this dose of antiserum, the following experiment (Table IV) is undertaken:

Diminishing amounts of human serum are mixed with a constant quan*tity of* complement and with the constant test dose of antiserum. At the

same time a control series of tubes is instituted, in which the antiserum is substituted by salt solution. After one hour of incubation at 37° erythrocytes and hemolysin are added. Table IV illustrates such an experiment.

TABLE IV.

Amounts of human serum in cubic centimeters.	Series A contains human serum +1 : 6×0.2 c.c.[1] Antiserum, +0.1 c.c. of guinea-pig's serum. One hour at 37°. +0.001 c.c. of amboceptor +1 c.c. of 5 per cent. ox's-blood.	Series B (control) contains human serum +0.2 c.c. physiological salt solution +0.1 c.c. guinea-pig's serum. One hour at 37°. +0.001 c.c. of amboceptor +1 c.c. of 5 per cent. ox's-blood.
	Hemolysis.	Hemolysis.
0.1	0	Complete.
0.01	0	
0.001	0	
0.0001	0	
0.00001	Slight.	
0	Complete.	

It is seen from the above table that 0.00001 c.c. of human serum still suffices to give a partial although incomplete fixation of the complement. The delicacy of the antiserum in this particular instance is not very great. In forensic practice, the reaction is carried out as shown in Table IV, but instead of the human serum, the solution of unknown blood stain in various dilutions is titrated. Control series B should not be omitted, because here, any foreign substance contained in the extract and which might interfere with the reaction can be detected.

[1] 1 : 6×0.2 c.c. means 0.2 c.c. of a 1 : 6 dilution.

CHAPTER XV.

PHAGOCYTOSIS. OPSONINS AND BACTERIOTROPINS.

1. Phagocytosis.

By phagocytosis is meant the taking up, or engulfing of foreign substances by certain cells (digestive cells or phagocytes) for the purposes of digestion. As a mode of nutrition, this is well known to exist, normally, in the lowest unicellular animals as for instance the amebæ. Intracellular digestion can, however, be traced to organisms higher in the scale of the animal kingdom, and even among mammals the function of cell ingestion is found, although limited in a sense, to a definite group of cells, especially those derived from the mesoderm.

The inspiration for the work on phagocytosis and the greater part of its theoretical considerations have emanated from Metschnikoff and his numerous pupils at the Pasteur Institute at Paris. Metschnikoff divides the phagocytes into two classes, the "sessile or fixed phagocytes," and the "wandering phagocytes." The first is the stationary endothelial lining of blood vessels, and lymph spaces, as well as the large cells of the spleen pulp and lymph glands; the second, consists of the white blood cells of the circulation. From another standpoint the phagocytes are divided into "microphages" and "macrophages." The former are practically identical with the neutro- and eosinophile polymorphonuclear leucocytes, while the latter present no distinct group, but include large lymphocytes, myelocytes, giant cells, etc. The cells designated as sessile phagocytes also belong to the class of macrophages. The size of the cell was considered by Metschnikoff as the deciding feature; not all macrophages are mononuclear as generally believed. Thus for example macrophages appearing in the peritoneal fluid of guinea-pigs frequently possess, like the giant cells of the tubercle, numerous nuclei. According to Metschnikoff it is primarily the microphages to whom the function of bacterial phagocytosis is allotted, while the macrophages serve for the purpose of ingesting dead or moribund tissue structure. Still there are certain pathogenic micro-organisms, tubercle bacilli, lepra bacilli, actinomyces, which are favored in being digested by the selective macrophages. The evidence of phagocytosis is established by mixing either in vitro or vivo the substance for phagocytosis, plus the phagocytes, and noting the changes which ensue; [either in a stained or unstained *preparation].* The phagocytes of the guinea-pig's peritoneal cavity are

especially well adapted for the study of phagocytosis in vivo. The following experiment of Metschnikoff may serve as a type.

A guinea-pig receives an intraperitoneal injection of goose's blood. Immediately following this, the leucocytes disappear from the peritoneal fluid. This is due partly to a destruction of leucocytes (Phagolysis) and partly because the leucocytes are repulsed and settle upon the peritoneal wall. In one to two hours this so-called negative phase is overcome and there is an increase of the leucocytes, especially of the macrophages in the exudate (Hyperleucocytosis). Now, the leucocytes can be seen sending forth short protoplasmic processes—pseudopodia, by means of which the erythrocytes are drawn into the phagocytes. After a short time the macrophages are filled with the erythrocytes. At first the ingested cells appear normal; gradually, however, they undergo changes, which are clearly visible in the unstained specimen, indicative of a disintegrating process, within the body of the phagocytes.

The same phenomenon as described for goose's erythrocytes can also be observed with bacterial bodies.

In order to exclude the possible bactericidal influences of the serum, it is advisable when one is working with bacteria which are readily destroyed as cholera vibrios, to previously induce a hyperleucocytosis in the peritoneal cavity. The guinea-pig receives an intraperitoneal injection of 10 to 20 c.c. of sterile bouillon or aleuronatsolution. In about twelve hours hyperleucocytosis takes place, and a capillary pipette inserted into the peritoneal cavity will withdraw a thick and turbid exudate.

If this animal is injected intraperitoneally with bacteria, and a smear of the peritoneal fluid made a short time after the inoculation, the bacteria will be seen lying within the microphages. This important fact has been variously interpreted. Pfeiffer and his pupils claim that the bacteria are first destroyed or their virulence greatly diminished by the bactericidal power of the serum and exudate, and that the phagocytes act only as receptacles for these already destroyed bacteria. Metschnikoff believes that the phagocytes take up the living bacteria and destroy them, thus representing these cells as the most important weapons of the organism in its protection against infection.

"Every time an organism that has lost its susceptibility toward a particular infective agent, either on account of a natural born immunity or an artificially attained one, comes into conflict with this infective agent, a struggle arises between the latter and the phagocytes of the threatened individual. It is the phagocytes that appear as victors, since they take up the bacteria into their protoplasmic bodies and digest them, thus forever depriving them of their power for evil." (Metschnikoff cited by Levaditi).

Critically considered, there can be no doubt that the phagocytes are in principle capable of dealing with living virulent bacteria. At the same time one must observe that the opsonins and bacteriotropins of the serum soon to be discussed, in most instances previously modify the living bacteria in some way at present still unknown. That, however, the phagocytes can ingest bacteria or protozoa which are alive and active, has been demonstrated

by Metschnikoff's school. Phagocytosis experiments were undertaken with motile bacteria and spirilla. On microscopical examination it was seen that a phagocyte was in the act of taking up a spirillum, part of which was engulfed by the cell while the remainder was still outside of the cell and continuing its active motility.

Not in all cases does phagocytosis of bacteria lead to destruction of the ingested microbes. More recently different experiments seem to prove that simple phagocytosis of bacteria must not be considered as identical with death of the same. Furthermore, the exudate from cases of anthrax in which the bacilli lie within the leucocytes, can still produce fatal anthrax when inoculated into animals.

Vital Staining with Neutral Red.

A more exact understanding of the bio-chemical nature of phagocytic digestion has been offered by the method of vital staining with neutral red.

Neutral red (used as a 1 per cent. solution in isotonic saline) is a chemical dye which stains only dead cells and not living ones. If live bacteria and phagocytes are brought into contact in hanging drop preparations (and a drop of the stain is added at various intervals to a different mixture), the first slide shows the extracellular living bacteria unstained, while of the intracellular bacteria, a part remains unstained and the other colored red.

The later the mixtures are stained, the more numerous are the intracellular red stained bacteria, showing that the injected micro-organisms remain alive for a short time, and then die. The intracellular bacteria retain their stain as long as the phagocytes themselves remain alive. Later, when the phagocytes die, the formerly red bacteria lose their stain. Metschnikoff's explanation of the red staining process is that during the act of digestion by the phagocytes, an acid ferment is liberated which gives the color reaction with the neutral red.

For many years Metschnikoff's phagocytic theory opposed the conception of Ehrlich and also Pfeiffer in relation to the importance of amboceptor and complement in the mechanism of immunity. It would be out of place here to review the various experiments performed and offered on each side in explanation of its standpoint. Suffice it to say that Metschnikoff denied the existence of free complement within the animal organism. He moreover claimed that the complement was found normally only in the phagocytes and hence called it "cytase," differentiating the two phagocyte groups as "micro- and macrocytase." The "cytase" is liberated when the phagocytes are broken up. The amboceptors are considered as split products of the phagocytes and known by Metschnikoff as "fixators."

2. Opsonins.

In recent years the closer relationship which has arisen between the followers of phagocytic and humoral theories was made possible by the fact that Denys and Leclef, Leishmann, Wright and Douglas and others, demonstrated that phagocytosis occurs in most cases only in the presence of serum. If the *phagocytes are* thoroughly washed, so that they are entirely serum-free,

phagocytosis will not take place, or will do so imperfectly. The belief of some authors that "spontaneous phagocytosis" without serum was altogether impossible, was disproved, especially by Löhlein. The manner in which the serum acts, whether it stimulates the digestive activity of the leucocytes or whether it so changes the bacteria that they can more readily be taken up by the phagocytes, has been settled in favor of the latter view through researches, especially of Wright and his followers as well as by Neufeld. The substances within the serum which thus modify the bacteria have been designated by Wright as "opsonins." ("opsono" = I prepare food for).

Opsonins are demonstrated by mixing bacteria, serum and washed leucocytes, allowing this mixture to remain in the incubator for a short time, and then staining smear preparations. Wright then counts a certain number of leucocytes and the number of bacteria found within these leucocytes.

The Opsonic Index.

The relation between this number of ingested bacteria and the counted number of phagocytes is designated as the phagocytic count. Wright compared the phagocytic counts of infected individuals with those of normal persons and found that those of the former were much lower. The relation existant between the two he expressed in the form of a fraction and that is known as the opsonic index. Thus a smear made from a mixture of equal parts of an emulsion of staphylococci, leucocytes and the patient's serum showed for example 75 cocci to 100 leucocytes; while one made from a mixture of equal parts of the same bacterial emulsion, leucocytes, but a normal individual's serum demonstrated 150 bacteria to 100 leucocytes. The opsonic index of the patient's serum would therefore be one-half (0.5).

According to Wright, the opsonic index expresses the animal's resistance against infection. He believes that a low opsonic index for a given bacterium indicates a susceptibility on the part of the individual for that particular infective agent. Furthermore, the opsonic index he claims can be used as an aid in the diagnosis of infectious diseases, inasmuch as opsonins are specific. Thus the opsonic index in a tuberculous individual is low only for the tubercle bacillus and not for other bacteria.

When an animal is immunized, its opsonic index toward the respective bacterium is considerably increased. The question has been asked whether the immune opsonins formed during this process are identical with the normal opsonins. Wright and a number of the more recent authorities believe that they are different. Neufeld, who discovered these immune opsonins independently of Wright, named them Bacteriotropins, and pointed out that while the normal opsonins are destroyed when heated to 56°, the bacteriotropins remain unharmed. As yet the exact nature of the immune as well as of the normal opsonins has not been clearly defined. It is still a matter for investigation whether in the case of opsonins one is dealing with entirely new

substances or whether they are the old well-known bodies like the agglutinins, complements and amboceptors with a new action.

The fact that the opsonic index is raised by immunization while it is usually found diminished during spontaneous infection in man, lead Wright to believe that good results may be obtained by increasing the opsonic index of the already infected individual by means of immunization. In this way he thought the patient's predisposition to the particular infection would be overcome, with the consequent obtention of the essential requirements for a cure. Wright's experiments showed that the opsonic index could be increased by injection of extremely small doses of dead bacteria (Wright's vaccines.)

Increase of Opsonic Index by Immunization.

If an individual suffering from an acne or furunculosis, and who has a low opsonic index for the staphylococcus, is injected with a very small number of staphylococci, his opsonic index sinks still more for a short period after the inoculation (negative phase). This is explained by the fact that the injected bacteria absorb the existing opsonins. New opsonins are however then produced, which immediately make up for the loss occasioned during the negative phase, with the result that after several days there is an increase of the opsonic index (positive phase) which lasts for a short time. Then the index again begins to fall, as the stimulus for the formation of opsonins is transitory. It usually sinks to below the normal level, only to rise again to a point slightly above the normal, where it remains stationary. This irregular curve represents the typical course of the opsonic content of the blood after a vaccine injection; apart from this characteristic picture numerous exceptions exist. Thus by the use of very minute bacterial doses, the negative phase immediately following the injection is entirely absent. Reversely very large doses exhibit a prolonged negative phase.

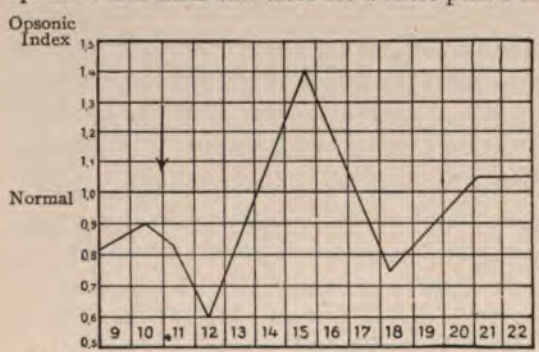

CHART 5.—Curve of the opsonic index following the inoculation of a small dose of staphylococcus vaccine. The arrow indicates the time of injection.

Wright graphically represents these variations in the opsonic index by charts, an example of which is given here (Chart 5).

In order that the therapeutic effect may persist, it is advisable to repeat the inoculation. A new injection should be given at the height of, or during the positive phase, as an inoculation repeated during a negative phase will result in further depression of the index unto a very low level. It is even possible in this way to harm the patient. The poor results obtained during the first era of tuberculin treatment can, according to Wright, be attributed to the failure of this observation. It is the production of cumulative positive *phases* that is the aim of vaccine treatment. (Chart 6).

Wright and his co-workers have noticed that an increase in the opsonic index usually runs parallel with an improvement in the condition of the patient.

Inasmuch as an increase in the opsonic index is occasioned by introducing into the general system even a very small number of bacteria, it seems probable that such spontaneous inoculation will take place during the course of an infectious disease. In fact, a spontaneous rise in the opsonic index is observed during convalescence or after the crisis of an infection. A high index is, however, also noticed at other times, for example tuberculous individuals show a higher index than normal persons. Wright explains this by the

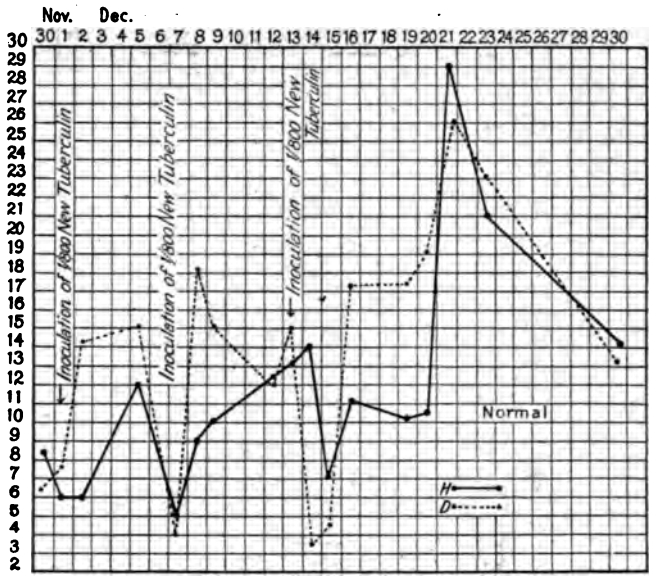

CHART. 6.—Opsonic curve during treatment with New Tuberculin.

so-called "auto-inoculation;" for example after moderate exercise, or work, tuberculin is liberated from the tuberculous focus and in this way acts like a therapeutic injection of tuberculin, *i.e.*, the index will be raised. Therefore, an excessively high opsonic index is of just as great diagnostic value as a low one. Wright furthermore believes that constant irregularities or variations in the height of the opsonic curve serve as plausible evidence for the existence of infection, because under normal circumstances the curve should remain at a level. Not infrequently, however, cases come under observation where in spite of a distinct evidence of the existence of an infection the opsonic index remains normal. In such instances for some reason, the bacteria and their products do not reach the general circulation and therefore no occasion is offered for either an elevation or sinking of the opsonic index. Wright and Freeman were able to show that all active and passive motions of an infected joint, as well as any vascular changes which induce a flow of lymph toward

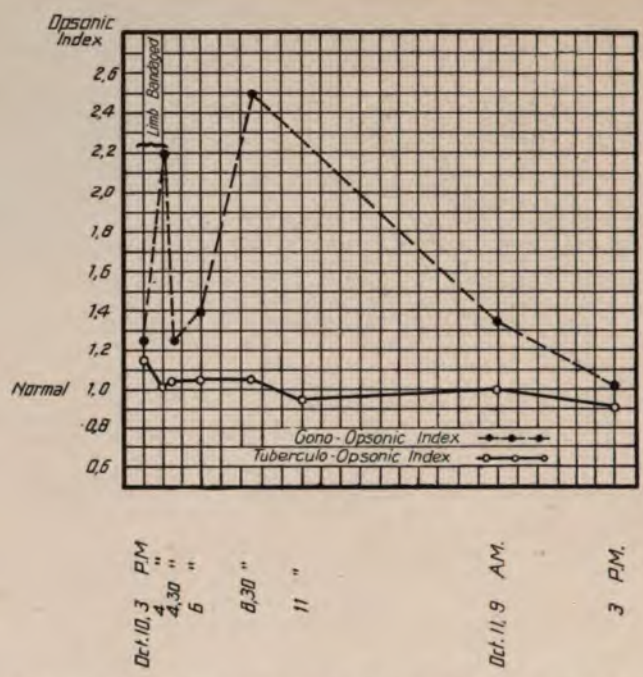

CHART. 7.—Increase in the opsonic index for the gonococcus brought about by Bier's Hyperemia.

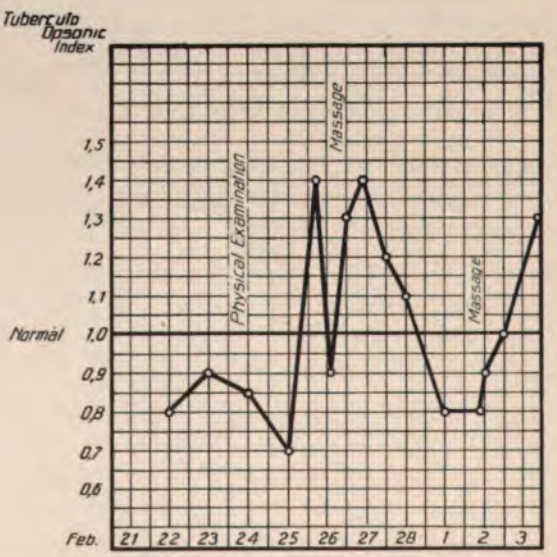

CHART. 8.—Tuberculin auto-inoculation following physical examination and massage. (*Tuberculous Lymph-adenitis.*)

the focus of infection, lead to auto-inoculations, which are manifested in a change of the opsonic index. Such artificial production of auto-inoculation can be employed in various forms as a means of diagnosis: thus, in articular rheumatism, massage; in pulmonary tuberculosis, breathing exercises; in laryngeal diseases, loud reading; and in tuberculosis of the lower extremities, active gymnastics will occasion changes in the opsonic curve.

An example is given in Chart 7. The patient was a woman with a swollen wrist joint. In order to decide whether this was a gonorrheal or tuberculous process, the opsonic index was taken and found to be 0.94 to 0.97 for the gonococcus and 1.03 to 1.35 for the tubercle bacillus. As these figures differed very slightly from the normal, the test was repeated, but this time after Bier's hyperemia had been applied and the forearm placed into warm water for one hour. The opsonic index for the tubercle bacillus remained the same, while that for the gonococcus had undergone marked variations.

A similar experiment with a woman having tuberculous lymphadenitis is given in Chart 8.

Wright makes use of these variations of index caused by auto-inoculation in determining the prognosis of a case. An infection is only then considered cured when artificial auto-inoculation is no longer possible.

The Technique for the Determination of the Opsonic Index.

For the determination of the opsonic index are required,

1. Serum of the patient.
2. Serum of the normal individual (as control).
3. Washed blood cells (Leucocytes).
4. Bacterial emulsion.

The blood serum is obtained from the finger tip at the root of the nail. It is most efficacious to first produce a hyperemia of this part by constricting

FIG. 18.

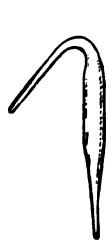

FIG. 19.

FIG. 20.

the finger either with a narrow gauze bandage or a small soft rubber tube (editor has found the latter much more convenient). The prick is then made with a needle or finely drawn out glass tube. The blood flows spontaneously and is collected into one of Wright's capillary tubes (Fig. 19) appro

mating the curved end of the latter to the blood (Fig.18). The straight capillary end of the tube (away from the blood) is then gently warmed in a small flame and sealed. The tube is laid down flat, and allowed to cool; in so doing the blood is sucked back from the unsealed capillary end; next this end may also be sealed in the tip of the flame. The blood then coagulates and the serum separates off. The separation of the latter may be hastened by centrifugalization for a short time.

In order to obtain leucocytes, a small test tube which holds 3 to 4 c.c. is filled 2/3 with a 1.5 per cent. solution of sodium citrate, and about 6 to 7 drops of blood from a healthy individual are collected into this solution (Fig. 21). The tube is inverted several times to thoroughly mix the blood so

FIG. 21.

FIG. 22.

that the citrate by precipitating the calcium salts of the blood, effectively prevents coagulation. The suspension is centrifugalized until the corpuscles are thrown down and a distinct white layer (leucocytes) is seen upon the surface of the red cells (Fig. 22). The clear supernant citrate solution is pipetted off, care being taken not to disturb the white layer. Some of the 0.85 per cent. saline is added, mixed and again centrifugalized. The washing with normal saline solution is repeated once or twice and as much of the clear liquid as possible is finally removed; the remaining cells are thoroughly mixed and in this form are ready for use.

The bacterial emulsions with the exception of the tubercle bacillus are made from agar cultures; the growths of gram + cocci may be as old as twenty-four hours, while the coliform organisms and the gram − cocci are preferable, if only four to ten hours old, the younger the better. A loopful of culture from an agar tube is thoroughly rubbed up with several drops of salt solution in a watch-glass by means of a small glass pestle. The salt solution is best added very gradually, drop by drop, thus making a more perfect emulsion. This may then be advantageously centrifugalized for a *varying period*, to bring down the large clumps. The supernatant opalescent

portion is taken off for use, thoroughly mixed, and if necessary diluted. Emulsions of coliform organisms are more easily made. Frequently it is sufficient to rub up with the platinum loop a loopful of such bacteria on the side of a small test tube containing saline. The proper thickness of the resulting emulsion varies. As a rule, bacillary emulsions are required to have a thicker appearance to the naked eye than coccal ones. The latter should be only slightly opalescent.

In order to make a satisfactory *tubercle emulsion*, a more elaborate method is necessary. The dead and dried tubercle bacilli are employed for this purpose. A portion of these bacilli is very thoroughly triturated in an agate mortar, or between two slides, or in a grinder devised for this purpose, at first alone and then with 1.5 per cent. salt solution added drop by drop. In this way a paste, and subsequently a comparatively thick emul-

FIG. 23.

FIG. 24.

sion is made. For use, a small portion of the resultant emulsion is centrifugalized until only the upper layers are fairly opalescent.

These upper layers are pipetted off, and thoroughly mixed. A smear of this should be made and stained in order to observe that the emulsion is free from clumps and not too thick. Such an emulsion sealed up in a glass tube and sterilized at 60° C. for 1 hour can be kept for about one week.

Streptococci may similarly be rubbed up in a mortar with 0.85 per cent. salt solution and then centrifugalized. As a rule, however, vigorous pipetting into a watch glass with subsequent centrifugalization for a few minutes is sufficient to remove the chains and leave a satisfactory emulsion.

If several specimens of blood are to be examined it is best to put up a "trial trip" and do a preliminary phagocytic count in order to test the strength and condition as regards clumping of the emulsion. The phagocytic count should be for tubercle between 1.5 to 2 per cell and for other organisms not less than 3 per cell. Accordingly, further dilution or concentration of the emulsion is necessitated. The pipettes employed for the opsonic index should be about 16 cm. long and made from glass tubing about 5/16 of an inch in diameter. They should all be approximately of the same caliber and but slightly tapering toward the point. The piece of tubing

FIG. 25.

should tightly fit the rubber nipple or bulb available. For use, the capillary end should be cut square and the pipettes marked with a paraffin pencil about 3/4 of an inch from their extremity. The content as far as this mark is the unit of volume in each case.

The rubber nipple is now held between thumb and forefinger and gently compressed, the capillary end introduced into the well mixed blood cells and the unit volume drawn up by slightly relaxing the pressure on the bulb. Next a tiny bubble is allowed to enter, then an equal volume of the emulsion, followed by another tiny bubble which latter is succeeded by an equal volume of serum. By gentle pressure on the bulb the several volumes are ejected upon a clean glass slide, and thoroughly mixed by alternately sucking the mixture into the pipette and squeezing it out again upon the slide. It is enough to repeat this action three times. Then the mixture is drawn up into the pipette, the end sealed in a small pilot flame, the pipette placed into

the opsonizer (Fig. 24) and the time noted. This operation is repeated with each serum.

Coliform organisms and the gram-cocci should be incubated not longer than six to eight minutes. Tubercle bacilli and other organisms require fifteen minutes more or less, according to the strength of the emulsion.

The pipettes are then withdrawn in the same order in which they were placed into the opsonizer. The contents of each are blown out on to a slide and very carefully mixed as before (Fig. 25). The entire quantity is divided between two or three slides and several smears are made, the best one being selected for counting. These slides should previously have been roughened with very fine (oo) emery paper, cleaned with a duster, and should rest on their concave surface so that the smear is made on the convex side. (It will be noticed that a slide can be made to rotate if resting on one surface (convex), but does not do so when resting on the concave surface). The smears are best made by means of the edge of a broken slide with a slightly concave edge. This "spreader" (Fig. 26) is made by sharply breaking a

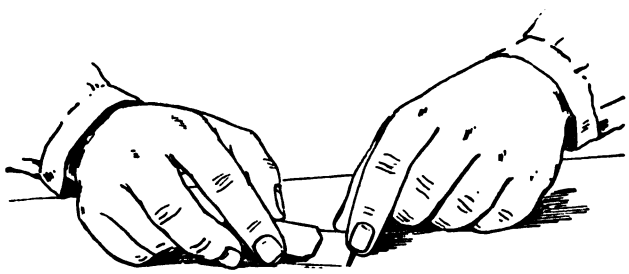

FIG. 26.

glass slide at about its middle, this being facilitated by scratching the edges of the slide with a glass cutter at the point where it is desired to break it. The editor has broken as many as twenty to thirty slides before a proper spreader was obtained. It pays to do this, because upon the sharpness of the fracture and cleanliness of the spreader depends the edge of the film, and secondarily the ease, rapidity, and accuracy of the count. If the film be well made, it will have a straight edge within which will be found practically all the leucocytes, as they are larger than the red blood cells, and therefore dragged to the end of the film.

The preparations are fixed in a saturated solution of corrosive sublimate for two or three minutes, washed with water, and stained with methylene blue or carbol-thionin (1/4 per cent. thionin, and 1 per cent. carbolic acid). Carbol thionin is by all means preferable. It should be slightly diluted and warmed before being poured upon the slide. Here it is allowed to remain for several minutes, then washed off in water, and the slide dried with filter-paper. The tubercle films are best fixed with formalin vapor, stained with hot carbol or aniline fuchsine, decolorized in 2.5 per cent. of H_2SO_4,

treated with 4 per cent. acetic acid to dissolve the erythrocytes and counterstained with 1/2 per cent. of methylene blue in 1/2 per cent. of sodium carbonate. It is most important that tubercle films be carefully stained because it is desirable to color every bacillus and yet not break up the leucocytes (Fig. 27).

With a 1/12 inch oil immersion lens a minimum number of one hundred polymorphonuclear leucocytes are now examined and the number of microbes they contain enumerated.

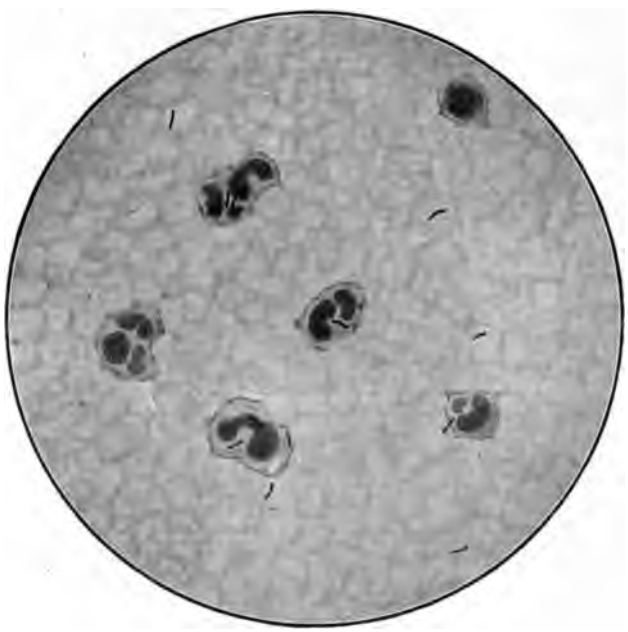

FIG. 27.—Phagocytosis of tubercle bacilli.

Similar calculation is undertaken with the normal control serum. The fraction obtained by dividing the number of bacteria contained in 100 cells, on the patient's slide, by the number in 100 cells, on the normal slide, gives the opsonic index of the patient's serum.

For example, the normal individual has 284 and the patient 262 bacteria in 100 cells, the fraction which gives the patient's opsonic index would be 262/284 or 0.92.

The principle of Wright's technique is simple, but it requires a great deal of practice before it is mastered. Only then are the results reliable. One must remember the same principles when counting the control slide as when the patient's film is counted. If in the last case, for instance, the cocci situated on the edges of the cells are not included in the count, the same should also be excluded in the first case. The absolute count is of no importance. It is the relative proportion which is significant.

As a normal control, it is best to take the average of the phagocytic

counts of a series (3 to 4) of normal sera or first equally mix the different sera, and take the phagocytic count of the pool.

Normal sera should not differ from one another in a tubercular opsonic estimation by more than 10 per cent.

Wright's Vaccine Treatment.

As has been said, the principle of Wright's vaccine treatment depends upon the immunization with small doses of dead bacteria, so-called vaccines, whereby the opsonic index of the individual is raised. This is usually associated clinically, with improvement in the patient's condition.

The effect of the immunization according to Wright depends upon:

1. Individual reaction of the patient.
2. Preparation of the vaccine.
3. Dosage and form of application.

The individual reaction of the patient can be measured by the opsonic index.

As for the preparation of the vaccine, Pasteur's contention that a vaccine must necessarily be made up of living cultures has not proved itself correct. Carefully killed cultures suffice in almost all cases. An example of the preparation of Wright's vaccine is here given.

The Preparation of a Staphylococcus Vaccine.

Agar cultures are grown for twenty-four hours, and about 3 c.c. of sterile normal saline solution is added to each culture. The growth is washed off into the saline solution by means of a platinum needle or freshly prepared capillary pipette. The suspension of bacteria is placed into a sterile tube, the end of this tube drawn out in the blow-pipe flame and sealed. The drawn out portion should be about 2 inches in length and as strong as possible. The emulsion is now vigorously shaken for fifteen minutes. The extremity of the drawn-out tube is then cut and a few drops of the emulsion expelled into a clean watch glass, or a small part of the drawn-off end is cut off so that a portion of the emulsion is still contained within it. The tube is resealed, and then submerged in water and kept at 60° for one hour. This usually suffices to kill the bacteria.

The small amount placed into the watch glass or in the capillary test-tube serves for the standardization, which is carried out as follows: A pipette and rubber bulb as prepared for the opsonic-index test, is also used here. A volume of freshly drawn blood of known corpuscular content, best taken from the worker's own finger, and an equal unit volume of bacterial emulsion is mixed thoroughly with six or seven volumes of 1 1/2 per cent. of citrate solution; several even films which may be fairly thick, are then

made by means of the ordinary edge of a slide, and stained with carbol-thionin, Leishman's or Jenner's stain.

The entire smear is divided up (with a blue grease pencil) into eight equal subdivisions, by one transverse line drawn parallel to the long diameter of the slide at its middle and five vertical lines, one at each edge of the smear, one in the center and one equally distant between the edge and the central line. It is also advantageous to employ an eye piece, the field of which has been divided or made very much smaller by the insertion of a small paper screen with a small central opening representing the size of the desired field. Five or six fields are then counted in each of the eight subdivided areas. The number of red blood cells seen in each field are enumerated in one vertical column, the number of organisms in the same field in another column. In this manner an average of the entire slide is obtained.

By means of a simple proportional sum, the number of bacteria per cubic centimeter of emulsion is estimated, *e.g.*, the number of red blood cells counted is 850 and the number of bacteria 1020. The red blood corpuscles used in the standardization are known to number 5,000,000 to a cubic millimeter or 5,000 million to a cubic centimeter; therefore the number of bacteria to a cubic centimeter of the unknown emulsion is expressed as follows.

850 : 1020 : : 5,000,000,000 : No. of bacteria per c.c. of emulsion,
$\therefore$ 6,000,000,000 = the number of bacteria per c.c. of emulsion.

After the emulsion has been heated for one hour, the tube is unsealed and a drop is expressed into an agar culture tube which is incubated for twenty-four hours to demonstrate whether the emulsion is sterile or not. At the end of this time, if a growth is observed, the emulsion must be heated again for one hour at 60° C. and its sterility again tested for.

Proper dilution of the emulsion is next undertaken. Small bottles containing 25 c.c. of 1/2 per cent. carbolic acid in sterile saline are aseptically closed with rubber caps; for example, it is desirable to make up these 25 c.c. with staphylococcus vaccine so that each cubic centimeter contains 500 million bacteria, then

$$\frac{\text{(desired amt. to each c.c.)}\quad 500{,}000{,}000 \times 25 \quad \text{(No. of c.c. desired)}}{6{,}000{,}000{,}000 \quad \text{(dose of original emulsion)}} =$$

2.08 c.c. or approximately 2 c.c. of the original emulsion must be added to the 25 c.c. (to be exact 23 c.c.) to make up the desired dilution.

The rubber cap is finally coated with melted paraffin wax.

For stock vaccines it is best to make up the different vaccines in the following concentrations:

1. *Staphylococcus* vaccine—prepared from various strains of staphy-

lococcus, aureus, citreus, and albus, in three concentrations: 1000 million, 500 million and 100 million, to the c.c.

2. Streptococcus vaccine in 20 m. 10 m. and 5 m. concentrations. Since the streptococcus grows very sparingly, cultures of two or three days growth may have to be employed for the preparation of a vaccine, and even then it may be necessary to use one broth culture instead of sterile salt solution to emulsify the agar cultures. On standardizing such thin vaccines it is frequently necessary to take one volume of blood to two, three, or even more volumes of emulsion and then calculate accordingly.

3. Acne vaccine in 20 m., 10 m. and 8 m.

4. Mixed acne in 20 m. acne and 500 m. staphylococcus.

5. Gonococcus vaccine in 50 m. and 5 m. Gonococcus vaccines are best employed as autogenous vaccines.

6. Typhoid vaccine in 1000 m. and 2000 m. for prophylactic inoculation.

7. Colon vaccine in 25 m., 10 m., 5 m. Vaccines of coliform organisms are very easily emulsified; as a rule they should not be older than twelve hours and not be sterilized for more than three quarters of an hour.

With the exception of the staphylococcus vaccines, it is advisable not to use stock vaccines, but autogenous vaccines, *i.e.*, vaccines made from the specific strain of bacteria causing the infection to be *treated*. It is very important to isolate the supposed pathogenic organism from the innocuous or less pathogenic bacteria contaminating or complicating the infection.

In tuberculosis Wright employs a dilution of Koch tuberculin (T. R.). Recently he has prepared a tubercle bacillus vaccine in the same way as the other bacterial vaccines.

The initial dosage varies with the different vaccines, but should in general be about 100 to 500 million of staphylococci where one may go as high as 2,500 or even 5,000 millions.

In colon, streptococcus, gonococcus and acne, doses of 1 to 3 million should be used at the beginning and then gradually increased.

In tuberculosis Wright starts with the T. R. in dilution equivalent to about 1/1000 mg. of the dry tuberculin substance and this is increased to about 1/600 mg.

Wright cites two general rules to be observed in the therapy of infectious diseases.

1. In all cases where the normal antibacterial power of the blood has been lowered, immunization is indicated.

2. Whenever the blood possesses strongly active curative powers, an increased blood supply to the infected part should be attempted in order that the antibacterial elements of the blood and leucocytes might display their effect. In such cases the production of hyperemia is particularly of help. Similarly, massage and other such therapeutic measures can be useful.

The therapeutic value of auto-inoculation is very slight and should not be encouraged, as in this way the exact dosage cannot be followed out.

Wright has employed these vaccines in staphylo, strepto, and gonococcus infections,

as well as in coli infections, tuberculosis, malta fever and carcinoma where injections of the bacillus neoformans Doyen were given.

From a critical review of the cases published, which were treated with vaccines by Wright and his fellow workers, one certain conclusion can be reached; namely, that given an infection, inoculations with small doses of the respective dead or extracted homologous bacteria, will result in a therapeutic immunization. Although Koch had advanced the same principle for the treatment of tuberculosis, it is Wright who first recognized the general application of this form of immunity. Furthermore, by means of his opsonic studies, he was able to prove that by the injection of even the minutest doses, for example 1/1,000,000 c.c. of tuberculin, immune reactions are incited.

In spite of this finding, investigators are still at variance over the question, and two camps exist: one of which believes that the ideal treatment of tuberculosis consists in the repetition of the small doses; the other, that the best results are obtained by gradually increasing the dose of tuberculin until very large doses are administered. Citron has found the latter course more satisfactory.

Since, as is known, tuberculin is one of the harmful agents in tuberculosis infections, it seems more advantageous to get the patient, if possible, into such a condition where he is able to neutralize large doses of tuberculin rather than to have him at a stage where even moderate doses suffice to give a reaction.

Other questions of importance in the vaccine therapy are: first, whether any parallelism exists between the increase in opsonic index and improvement in the clinical manifestations; second, whether the opsonic index must necessarily be used as a guide in vaccine treatment.

As to the first, Wright has pointed out in numerous cases on record, that exact study has proved that such parallelism exists. This fact is probably correct in the majority of instances, but it cannot be considered as an infallible rule, inasmuch as the formation of opsonins is only one of a great number of factors in the complicated process of healing, and consequently one should not be surprised when in some instances in spite of a rising opsonic index, the patient's clinical condition becomes worse, and reversely where improvement occurs although the opsonic index does not change.

Accordingly, the value of the opsonic index during the course of treatment becomes secondary in importance to the exact clinical observation of the case. Wright and his school have shown that certain bad effects may follow from the injection when performed during the negative phase. With the use of small doses the negative phase becomes short—only one day or even less; accordingly it is very probable that this state is entirely passed when an injection is repeated on the fifth to eighth day.

The tuberculin therapy at the Kraus clinic is conducted on this principle, *without* estimation of the opsonic index. And yet, no harmful effects have

ever been noted; while general improvement, as increase in weight, diminution in temperature and cessation of cough, are constantly observed. It would be illogical to neglect these clinical data and give preference to the hypothetical action of opsonins as a guide in treatment.

It seems that Wright himself does not insist as strongly as before upon the determination of the opsonic index. One of his assistants, Matthews, has recently made the statement that in a great number of cases the determination of the opsonic index is entirely out of the question. If the choice between injections without estimation of said index and entire omission of inoculation should arise, therapeutic inoculation without the index is by all means indicated. Although it is almost a general tendency at present to omit the opsonic index in the treatment of staphylococcus infections, this may at times also hold good in tubercle, gonococcus and streptococcus infections as well as in prophylactic typhoid inoculations.

CHAPTER XVI.

Passive Immunization (Serum Therapy). Bacteriolytic Sera. Serum Sickness. Anaphylaxis. Special Serum Therapy.

In the former chapters it was learned that during *active immunization* specific protective bodies were formed which circulate in the blood and can, by means of the serum be transferred to another organism. By animal experimentation it was further found that such bodies exert this protection against fatal intoxication or infection in various ways; thus, as antitoxins and antiaggressins they neutralize toxic poisons and aggressins; as bacteriolysins they bring about lysis of the bacteria; while as bacteriotropins they prepare the bacteria for phagocytosis. The defending qualities of such a transferred serum is evident not only if the infection is incited at the same time as, or a short period after the serum is given, but in numerous instances curative effects are observed if the serum is given even after infection has already taken place.

Of all sera, those with *antitoxic properties* have met the greatest success in therapeutic application. They have already been referred to in their respective chapters.

The efficiency of the pure bacteriolytic sera on the other hand has been disappointing. The reasons given for this lack of curative action is, in the first place, *the inability of bacteriolytic serum to neutralize the endotoxins.*

Pfeiffer's experiment revealed that if the number of bacteria exceeded a certain limit, then in spite of bacteriolysis, death of the animal takes place. This was explained by the existence of endotoxins. By bacteriolysis the endotoxins previously found within the bacteria are liberated and thus get a chance to become toxic.

The aim therefore, was to produce antiendotoxic sera. This was, however, precluded from materializing by the erroneous view of Wolff-Eisner who claimed that it was impossible to immunize against endotoxin.

Numerous methods have been advocated for the liberation of these endotoxins: maceration of bacteria, exposure to very low temperature, admixture with chemical substances which would dissolve the outer capsule, ferment digestion, growth upon certain culture media, etc. At the present day, there is absolutely no doubt that the bacterial bodies contain poisonous substances against which it is difficult and to a certain degree impossible to attain an immunity.

Whether one should adhere to the old idea and apply to these the term, endotoxin, or include therein the class of true toxins with the only difference that they are not secreted but contained within the bacterial body and therefore more difficult to isolate, is purely a question of theoretical importance.

Another cause for the therapeutic failure of bacteriolytic serum, is given by Bail and his school, as the lack of its antiaggressin action. This applies only to the cases in which the bacteriolytic serum was produced by immunization with dead bacteria.

When live bacteria are used, this objection is not to be considered, as according to the experiments of Wassermann and Citron, "aggressin" is nothing more than the immunizing substance of the living bacteria. As for the structure of the antiaggressins, the author was able to show that like the bacteriolysins, they are amboceptors which bind complement.

Artificial aqueous extracts of living bacteria belonging to the class of half parasites made according to the method of Wassermann and Citron, contain the endotoxin as well as the aggressin. Such artificial aggressins, therefore, represent ideal antigens. The sera produced by their injection contain but few bacteriolytic bodies and a very large number of amboceptors, easily demonstrable by the Bordet-Gengou reaction.

Wassermann explains the lack of therapeutic efficiency on the part of the bacteriolytic sera, by the absence of complement of the organism, as well as by the inability of human complement to fit all animal amboceptors. As is known, amboceptors increase during immunization while the complement content remains the same. But since amboceptors without complement remain inactive, even a very strong serum may only be slightly effective, depending upon the amount of existing complement. If too many amboceptors are injected, the serum may become entirely powerless due to a phenomenon similar to Neisser and Wechsberg's complement deviation. Wassermann advises therefore the addition of complement to a serum before its injection, in order to activate it. This suggestion has not been widely adopted in practice.

It is for a similar reason, that the classical experiment of bacteriolysis is so beautifully demonstrable in the guinea-pig's peritoneal cavity, an area relatively poor in cells, while this phenomenon is incomplete and replaced by phagocytosis when occurring in the blood, inner organs, and subcutaneous connective tissue. It is in this connection that Metschnikoff and his followers see the main reason for the failure of the therapeutic activity of bacteriolytic sera.

An additional impediment is offered by the wide differences which exist between the numerous strains of the same bacterium. This may be so marked that an immune serum produced with one strain will enfold no protection against a different strain of the same bacterium. It is now overcome to a certain extent by immunization with as many different strains of the same bacterium as possible (polyvalent sera).

Cultures grown upon artificial media for a very long time, adapt themselves entirely to their new surroundings and frequently lose some of their biological characteristics, *e.g.*, virulence. If the culture is then inoculated

into an animal, the virulence is usually increased thereby only for the respective animal species, but may at the same time be lowered for man.

Many authors, therefore, employ for the production of immune sera only virulent strains of bacteria freshly isolated from man.

In spite of all the above considerations, the fact still remains that most immune sera excepting those of the cholera, typhoid, and paratyphoid bacteria, show no bacteriolytic tendencies even under the most favorable circumstances; but by means of their amboceptors they fix free complement and with the aid of bacteriotropins, stimulate phagocytosis.

Whether complement fixation is at all to be considered as a protective phenomenon, cannot with the presently existing evidence be definitely decided.

Conditions are much more favorable as far as the bacteriotropins are concerned. Active phagocytosis is always an expression of good resistance power. It is not necessary for the leucocytes to digest the bacteria; it is amply sufficient if a protective wall of these cells is formed (Ribbert, Citron, Gruber); moreover they can neutralize the bacterial poisons. In this connection it must always be borne in mind that phagocytosis by no means necessitates the death of bacteria.

Granting, however, that all the above requirements have been fulfilled and a suitable serum has actually been produced, will such a serum always be effective, or are there any other causes which may interfere with its good results? In order to answer this, the infectious diseases must be divided into acute and chronic. With the first class, success is quite assured as long as it is possible to bring sufficient amounts of the active serum substances into direct contact with the bacteria. In meningeal infections, intraspinal injections may have to be adopted. It is difficult, however, in cases of this nature to judge definitely whether the serum therapy was really the effective agent, inasmuch as diseases like erysipelas, meningitis, pneumonia, etc., are self limited, lasting for a period of time and then subsiding of their own accord.

With the chronic infections, on the other hand (especially tuberculosis), serum therapy has a new difficulty to overcome. As a result of the long duration of the disease, it is naturally impossible by means of a single injection to introduce sufficient curative bodies, as can be accomplished in diphtheria, for example. It is necessary, therefore, to repeat the injections for a long period of time. Under such conditions the human organism produces antibodies against the foreign proteid, perhaps even against the curative substances in the serum (antiamboceptors). In both instances the desired effect of the serum is lost.

The interaction between the injected serum and the bodies produced by the organism immunizing itself against it can manifest itself in various clinical symptoms known as the "hypersusceptibility" reaction, or the "serum

sickness," carefully studied by v. Pirquet and Schick. The evidences of serum sickness are numerous. Those present most frequently are fever, skin eruptions, swelling of the joints, glandular enlargement and edema.

These symptoms may follow even the very first injection of serum. They develop as a rule, after an incubation period of eight to ten days; slight reddening at the point of injection accompanied by moderate swelling of the regional lymph glands, appear as prodromal manifestations.

The general condition of the patient is generally only very little disturbed, in spite of the frequently associated high fever. Still there are instances, especially after the introduction of large amounts of serum, where the symptoms continue for about four to five weeks and then lead to severe disturbances.

The associated skin eruptions above mentioned, are usually of the type of an urticaria; although Hartung describes rashes simulating scarlet and measles.

As the most positive symptoms of serum sickness, v. Pirquet and Schick consider the following:

1. The occurrence of the exanthema seven to fourteen days after injection.
2. First appearance of the rash around the point of injection.
3. Regional enlargement of the lymph glands.
4. Complete absence of any changes in the mucous membranes.

Measles is excluded by the non-presence of Koplik spots, coryza, and conjunctivitis.

In scarlet fever the following symptoms help to exclude serum sickness:

1. Initial vomiting.
2. Occurrence of angina before or at the same time as the exanthema.
3. High fever.
4. The simultaneous existence of the infection among others in the hospital or neighborhood.

If the serum disease does not arise after the first, but after a later injection, it is characterized by the absence of, or very marked diminution in the length of the period of incubation, and in addition by increased severity of the symptoms.

Anaphylaxis.

Serum sickness belongs to a group of conditions designated by the terms "anaphylactic" or "hypersusceptibility" phenomena. The subject of anaphylaxis is one of present interest and its importance is manifest not only in serum sickness and in the tuberculin reaction, but in a great number of previously unexplained clinical occurrences. Only few of the most important experimental observations upon which this study is based, can here be reviewed. Those of Arthus and Theobald Smith deserve special consideration.

The Arthus Phenomenon.

If a rabbit is injected subcutaneously with horse's serum at intervals of six days, a soft infiltrate which remains for two to three days appears at the site of injection after the fourth inoculation, a harder infiltration which continues for a longer period of time after the fifth inoculation, and gangrene after the sixth or seventh. A rabbit injected subcutaneously for a long period of time, on receiving an intravenous inoculation of horse's serum, may die with severe general symptoms several minutes after the latter injection.

The Theobald Smith Phenomenon.

Theobald Smith observed that guinea-pigs injected with neutral mixtures of diphtheria toxin and horse's antitoxic serum would be killed if after an interval of several weeks they were given a subcutaneous injection of normal horse's serum (several cubic centimeters).

Otto and others showed that both of these phenomena, above described, are identical in their principle; thus, that of Arthus can be likewise induced after a single injection of horse's serum if the first dose is small, and if the interval between the first and second inoculation is sufficiently long (about three weeks or more).

Anaphylaxis is specific; that is the animals made anaphylactic against horse's serum will produce a reaction only, when subsequently injected with horse's serum and not when any other, like bovine serum is used. Even a single injection of 0.001–0.004 c.c. of horse's serum suffices according to Rosenau and Anderson, to produce anaphylaxis in a guinea-pig. The greater the amount of serum given at the first inoculation, the longer is the period which must elapse before the onset of the state of hypersusceptibility. With doses of several cubic centimeters, this interval is two to three months in duration.

The effect of the second injection depends largely upon the method of its administration. Given subcutaneously or intraperitoneally, 5 to 6 c.c. are required to bring about acute death of the animal, while by the intravenous or intracerebral route fractions of a cubic centimeter usually suffice.

Antianaphylaxis.

Animals which recover from their anaphylactic condition after the second injection, become antianaphylactic, *i.e.*, they do not react to further injections of the same serum or proteid solution. Such immunity appears two hours after the recovery from the anaphylactic shock.

In order to prevent anaphylaxis in animals, Besredka and Steinhardt advise the use of a very small amount of serum for the second injection, followed by a larger dose in twenty-four hours; or an injection of a very large dose during the period of incubation, best on about the eighth day.

Passive Anaphylaxis.

Anaphylaxis like immunity can be transmitted from one animal to another by means of the serum. Passive anaphylaxis is best demonstrated by injecting the anaphylactic serum subcutaneously, followed in twenty-four hours by the inoculation of the respective antigén.

No absolutely decisive explanation has as yet been offered for the anaphylactic status. It seems certain, however, that its phenomena are closely associated with the process of immunity.

Since the term immunization usually implies a beneficial process, while anaphylaxis in most instances represents a situation of an injurious nature, v. Pirquet recommended the term "allergie" to designate the reactive changes which an organism generally exhibits after infection or injection of an antigen. The "allergic phenomena" are divided into those associated with diminished susceptibility, *i.e.*, prophylaxis; and those with increased sensitiveness, *i.e.*, anaphylaxis.

Besredka adheres to the view that the anaphylactic syndrome especially expresses an *insult* to the central nervous system. He was able to show that susceptible guinea-pigs when etherized, will bear the second inoculation of the serum perfectly well.

v. Pirquet and Schick, Friedberger and others, consider the precipitin action as the

basis for the anaphylactic phenomena. Other etiological factors, also come into consideration; such as the increase of the sessile receptors with simultaneous absence or marked diminution of free antibodies, and the absorption of the complement by the amboceptors, in vivo.

Attempts have been made to employ the specificity of anaphylaxis for diagnostic purposes, but as yet the results obtained do not justify the clinical consideration of the methods.

Special Serum Therapy.

Meningococcus Immune Serum.

1. *Meningococcus Serum.*—Numerous investigators have attempted the production of an immune serum for man, among these Jochmann, the Berlin Institute for infectious diseases, Ruppel, Kraus, Flexner and Jobling, and others. The sera of Jochmann (Merck) and Ruppel (Höchst) are produced by immunization of horses with meningococci which are at first employed in dead, and later in live form. The other mentioned sera are attained by immunization with bacterial extracts or bacterial extracts plus full bacteria, and therefore contain agglutinins, precipitins, bacteriotropins, amboceptors and antiendotoxins. It is difficult to test the efficiency of these sera in animals, as the meningococci vary greatly in their virulence towards them. Jochmann and Ruppel assert that they have been successful in growing cultures extremely virulent for animals, which they employed for the titration of the therapeutic value of the serum. In the institute for infectious diseases, the method of complement fixation is employed for the titration of the therapeutic value of the serum. This procedure is very unreliable. The protection of the serum in mice against the meningococcus endotoxin as well as the demonstration of the bacteriotropic action of the serum is far more significant.

In man, the immune serum is injected intraspinously, after a quantity of spinal fluid has been withdrawn to relieve the pressure. In adults 20 to 40 c.c. and in children 10 to 20 c.c. are daily injected until either clinical improvement or a fatal prognosis becomes manifest. It is advisable to precede the serum inoculation by a morphine injection, and to elevate the pelvis for eight to twelve hours after the inoculation. *The earlier the serum therapy is instituted, the more favorable are its results.* Subcutaneous applications of the serum or employment of a serum older than three months is absolutely of no use.

Both in the United States and in foreign countries the value of the serum as a therapeutic agent seems fairly established. In Germany, the serum is obtained gratis at the institute for infectious diseases at Berlin. The serum in Switzerland is distributed by the serum institute of Bern (Kolle). In the United States, Rockefeller's Institute in New York first conducted its dispensation, but now it is under the supervision of the New York Board of Health.

Numerous statistics can be cited exemplifying the good results of the serum. The following figures given by Levy describing the experiences of the Essen epidemic are especially instructive:

From the first of January until the first of November, 1907, the total number of epidemic meningitis cases which occurred in Essen were:

55 Cases with 29 Deaths = 52.72% Mortality,

of these, treatment was given outside of the barracks to

15 cases with 12 deaths = 80% mortality,

inside the barracks were treated

40 cases with 17 deaths = 42.5% mortality,

of these

14 cases were not treated with serum with 11 deaths as a result = 78.6% mortality,

those treated with serum were

23 cases with 5 deaths = 21.7% mortality,

of these, those which were treated only incompletely (subcutaneously) and with insufficient doses, numbered

6 cases with 3 deaths as the outcome = 50% mortality,

systematic intraspinous treatment with large doses.

17 cases with 2 (1) deaths = 11.8 (6.3)% mortality.

The figures in parenthesis represent the moribund cases coming under treatment and the percentage which would result if these were not included in the calculation.

The experiences with the serum of Flexner and Jobling are similarly encouraging. In a report of 400 cases the mortality is reported as lowered from 80 per cent. to 20 per cent.

2. *Streptococcus Immune Sera.*—The rôle of the streptococcus in some diseases, for example, scarlet, is imperfectly understood. Moreover it has only been indefinitely established whether there are various groups or only one kind of streptococcus; even the significance of their virulence or hemolysin formation is not clear. Such are the difficulties which account for the great number of methods advocated for the production of an immune streptococcus serum. The oldest serum of the many, is that of Marmorek. It was produced by immunization with a strain made highly virulent by passage through animals.

The various other forms of the sera on the market are:

a. Serum Aronson (Schering).—This is a polyvalent serum produced by immunization of horses with cultures pathogenic for man; some strains having previously been passed through animals, others not. The strength of the serum is tested in mice infected with the latter strains.

b. Serum Meyer-Ruppel (Höchst Farbwerke).—Horses are first immunized with a strain of streptococcus whose virulence has been raised by passage through horses and mice; each horse is then injected with a different strain of human streptococcus. When the serum of each animal is of such a strength that doses of 0.01 to 0.0005 c.c. protect mice infected with its own particular strain, the sera of the different horses are mixed. Thus a polyvalent serum is obtained.

c. Serum Menzer (Merck) is monovalent and produced by immunization with a culture which is pathogenic for man and not passed through animals.

d. Serum Moser is polyvalent, produced by injections of streptococci from scarlet fever. The sera of Menzer and Moser are not tested by injections of white mice. The others are. One cannot strictly rely upon this method of serum titration for its employment in man. The virulence of streptococci against mice and human beings bears no definite relation. A serum may be perfectly efficient in mice both for prophylactic and therapeutic purposes, and be entirely inactive in man; also *vice versa*. The action of the serum should be in the main of bacteriotropic nature.

Antistreptococcus serum has been tried in scarlet fever, puerperal sepsis, erysipelas, and articular rheumatism.

Recently complement fixation experiments (Foix and Mallein, Schleissner) have shown that the streptococci of scarlet fever can be definitely separated from the other varieties of these bacteria.

In this disease favorable results have been observed by the use of Moser's serum.

Escherich states that of 112 scarlet fever cases injected, those receiving the serum on the first and second days of their illness all recovered while of those injected later on, there was a high percentage of mortality. Other authorities have seen no or only very slight effect from the serum treatment.

Two hundred cubic centimeters of Moser's serum must be given subcutaneously.

The treatment of puerperal fever has been favorably influenced by Aronson's and Meyer-Ruppel's serum, of which 50 c.c. are injected on several successive days.

Menzer's serum is said to serve its purpose best in acute and chronic rheumatism as well as in tuberculous mixed infections.

In erysipelas all the different well known sera have been employed. On account of the very variable course of the disease it is difficult to judge the exact value of the serum employed. In fact, thus far one cannot with certainty depend upon any serum treatment of a streptococcus infection. The serious nature of such infections, makes every possible therapeutic measure strongly justifiable.

Pneumococcus Immune Sera.

3. The pneumococcic sera most frequently used are those of Pane, Römer and Merck. Pane immunizes donkeys with highly virulent pneumococci and uses the serum for the treatment of pneumonia. Several Italian investigators record favorable results.

Römer prepares a polyvalent serum by injecting horses with different strains of pneumococcus obtained directly from man; the strength of the serum is tested in mice. The serum is mainly employed both for the protection and cure of ulcus cornea serpens.

The result according to Römer depends upon the very variable virulence of the pneumococci. The severity of the infection in man is said to run parallel with the virulence in mice. Römer, therefore, ascertains in every case of ulcus serpens whether his serum has any protective bodies for that particular strain of pneumococcus, and tests the virulence of the same.

The serum can be injected intravenously and subcutaneously, and in pneumococcus meningitis, intradurally. It is manufactured by the Höchst Farbwerke, in vials of 10 and 20 c.c.

A similar serum is manufactured by Merck. It is obtained from horses and standardized at the Institute for experimental therapy in Frankfort o/M. so that 0.01 c.c. injected subcutaneously protects a mouse inoculated intraperitoneally twenty-four hours later with 10 to 100 times the lethal dose of a living pneumococcus culture. This is known as a normal serum and one cubic centimeter contains one immunity unit (I. E.). The serum on the market contains 20 to 40 units per c.c.

In pneumonia 200 to 400 units are given subcutaneously and repeated in three to four days, if the fever does not subside. As a prophylactic inoculation 200 to 400 units are given to old people where a "hypostatic" pneumonia is feared. In ulcus serpens of the cornea 200 to 400 units are employed and if no improvement sets in, the dose is repeated upon the third day. In addition, several drops of the serum are instilled into the conjunctival sac every two hours. As a prophylactic dose in this disease 100 units suffice.

Merck also prepares a vaccine of dead pneumococci in doses of 1 c.c. which further aid in the treatment of pneumococcic infections. 1 c.c. of such dead pneumococci can be administered for the prophylaxis of ulcus serpens.

4. *Pest Sera.*—A large number of pest sera are in use.

a. The Paris serum (Yersin) produced at Pasteur Institute by immunization of horses with dead and later on living bacilli.

b. The Bern serum of Tavel employs the same principles.

c. Lustig's Serum.—For this serum, horses are immunized with the pest-nucleoproteids. Pest cultures are broken up by 1 per cent. of potassium hydroxide and from this, by the addition of acetic acid, the nucleoproteid is precipitated and then suspended in salt solution to serve as antigen.

d. Serum of Terni-Bandi is prepared by the immunization of donkeys and sheep with natural pest aggressins.

e. Serum of Markl is supposedly an antitoxic serum prepared by immunization with filtrates of old pest bouillon cultures.

All the above sera contain agglutinins, precipitins, bacteriotropins and amboceptors; the serum of Terni-Bandi contains aggressin amboceptors, that of Markl, antiendotoxins.

The sera are tested for their anti-infectious properties in animals such as guinea-pigs, rats, mice. Markl also estimates the toxin neutralization power of his serum.

The Paris serum comes either in dry form or in bottles containing 20 c.c. without any preservatives. Ten to 20 c.c. should suffice as a prophylactic injection, although Martini advises 100 c.c. at least. The period of protection is short, averaging about fourteen days.

Prophylactic injection is advisable in those instances where an immediate protection is necessary, like the inoculation of physicians and nurses attending pest patients. Under all other circumstances either active immunization or the simultaneous method of Shiga should receive the preference.

For the treatment of pest infections, Calmette and Salimbeni advise intravenous administration of 20 c.c. and two subcutaneous injections of 40 c.c. each—all to be given on the first day; on the second day two similar subcutaneous injections; and if the case is of a severe nature, the dose may be doubled. The results are variable.

From comparative studies, it seems that Lustig's serum is somewhat weaker than the Paris serum. The sera of Terni-Bandi and Markl have not been sufficiently employed, so that opinion is reserved.

5. *Tuberculosis Sera.*—The best known and most studied are those of Maragliano and Marmorek.

a. Serum Maragliano is prepared by Maragliano's institute in Genoa from horses which are immunized for about six months with the soluble substances of tubercle bacilli. The favorable action of the serum is reported on, especially by Italian authorities.

b. Serum Marmorek is prepared in the laboratory of Marmorek, at Paris-Neuilly, by the immunization of horses with the so-called "primitive" tubercle bacilli, *i.e.*, young tubercle bacilli whose acid fast character is still very slight or entirely absent. When the horses have attained a high grade of immunity, they receive injections of various strains of pure cultures of streptococci obtained from the sputum of tuberculous patients. The serum of these animals is, therefore, antituberculous and at the same time polyvalent antistreptococcic (a double serum), serving against the mixed infections.

This serum is administered daily, either subcutaneously 5 to 10 c.c. or per rectum 20 c.c. The latter form is more advisable for the sake of preventing anaphylaxis. Citron has found the serum entirely harmless, the bad effects described by some being probably due to the idiosyncrasy of patients against foreign sera. The most favorable results have been claimed as found in localized bone and joint tuberculosis and in the incipient stages of pulmonary tuberculosis. Especial consideration of the serum should be given in those patients who evince persistent temperature or the very severe but not hopeless cases, where the tuberculin therapy cannot be undertaken. In some of these instances very encouraging results have been noted.

Occasionally the author started with the serum treatment, and then combined with it the tuberculin administration and finally left the serum away entirely.

6. *Anthrax Sera.*—Sclavo, Deutsh, Sobernheim and others have produced immune sera by the immunization of donkeys, sheep and horses. These have been mainly employed in veterinary practice.

In man the serum has been tried only by Sclavo. He injects 30 to 40 c.c. subcutaneously for several successive days; in severe infections 10 c.c. are administered intravenously. Two cases described by Bandi received 150 c.c. intravenously.

7. *Typhoid Immune Sera.*—The ordinary bacteriolytic sera (Tavel) have not met with the desired success in the therapy of typhoid fever. Attempts have, therefore, been made to produce antiendotoxic sera. Chantemesse treats horses for several years with bouillon filtrates; Besredka injects first dead and then living typhoid bacteria from agar cultures, Mac Fadyen breaks up the bacteria at very low temperatures and thus liberates the endotoxin for purposes of immunization. Kraus and von Stenitzer use bouillon filtrates and aqueous bacterial extracts as is likewise done by Meyer-Bergell and Aronson. Garbat and Meyer employ sensitized typhoid bacilli, *i.e.*, bacteria united with their bacteriolytic amboceptors.

Chantemesse injects several drops of his serum subcutaneously. The action lasts ten days. Only occasionally is a second inoculation necessary; if so, it must be much smaller. His results have been good and have mainly depended upon an increase in the opsonic index.

Meyer and Bergell as well as Kraus give 20 to 50 c.c. subcutaneously.

8. *Cholera Serum.*—Similar attempts for the production of a cholera antiendotoxic serum have been made. Kraus has succeeded in obtaining an antitoxin against some El-Tor vibrios which have all the characteristics of true cholera vibrios.

The experiments with Kraus' serum, and Kolle's serum (Bern Institute), at present being conducted in Russia, seem to be favorable.

The serum therapy of infectious diseases is still in its primitive stages. The contradictory results of many authors are to be associated not only with the variable efficiency of the sera, but also with the method, the time, and the dose chosen for administration.

The same serum in the hands of different physicians may yield opposite results. These subjective sources of error must be overcome, or minimized by making a complete and thorough study of the effects which a certain serum may have and actually does have; here all the clinical and laboratory guides must be made use of. Employed in this manner, serum therapy will even at the present stage lead to beneficial results.

Wright's motto at the beginning of his book on vaccines "The physician of the future will be an immunisator," can justly be reversed to read, "the immunisator of the future will be a physician."

Fig. 1. Positive v. Pirquet Reaction
(Original drawing)

a b

Fig. 2. Ophthalmo-reaction
(Original drawing)

a) Control eye b) Reaction of 1°—2° grade

Fig. 1. Very strongly positive Wassermann Reaction (++++)

Fig. 2. Strongly positive Wassermann Reaction (+++)

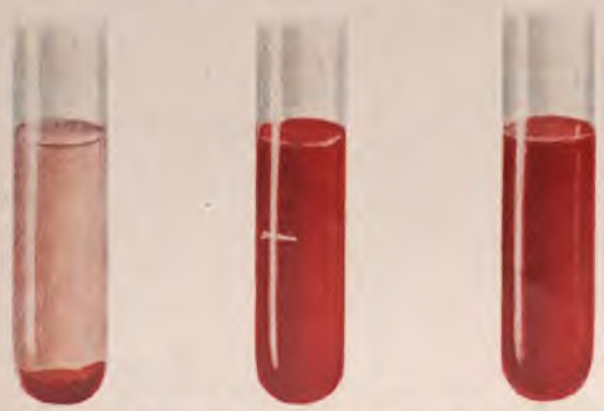

Fig. 3. Positive Wassermann Reaction (++)

a

Fig. 7

a) after 24 hrs

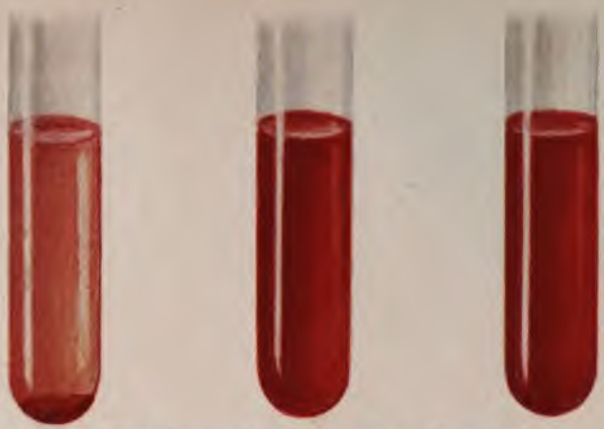

Fig. 4. Weakly positive Wassermann Reaction (+)

Fig. 5. Doubtful Wassermann Reaction (±)

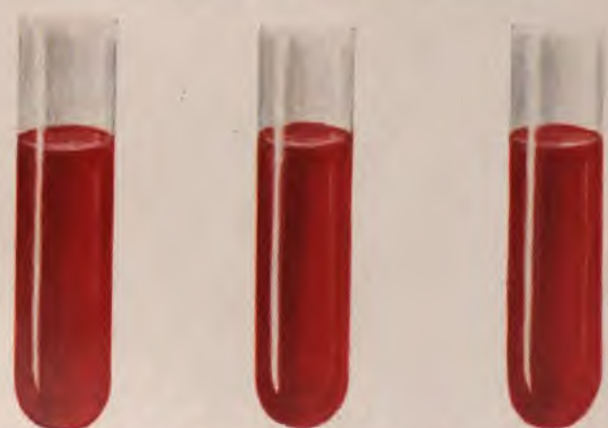

Fig. 6. Negative Wassermann Reaction (−)

ıan shaken up.

SUBJECTS, INDEX.

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